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THE UNIVERSITY OF ALBERTA

OXYGENASE ACTIVITY OF SOME PEROXIDASE ENZYME SYSTEMS

by

IAN DAVID MACDONALD

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA
SPRING 1989

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Gustav Graff
Gustav Graff, Ph.D.
Research Associate
1/18/89
Date

Linda A Anderson
Linda A. Anderson
Biochemist II
1/18/89
Date

kb

APPROVAL: Carl D. Lunsford
Carl D. Lunsford, Ph.D.
Senior Vice President
Research & Development Division

DATE: 1/28/89

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Cornelia Bohne
Division of Chemistry
National Research Council of Canada
Ottawa, Ont. K1A 0R6

Canada

University of Alberta
Edmonton
Canada T6G 2G2

Department of Chemistry
Faculty of Science
E3-44 Chemistry Bldg., Tel. (403) 492-3254 Fax (403) 492-8231

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(Signed) I. David MacDonald

Permanent Address:

85 Highland Avenue
Charlottetown, P.E.I.
Canada
C1A 2H7

Date: December 16, 1988

THE UNIVERSITY OF ALBERTA
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The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled "Oxygenase Activity of Some Peroxidase Enzyme Systems" submitted by Ian David MacDonald in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry.

H.B. Dunford
H.B. Dunford, Supervisor

Josef Takats

Brian D Sykes

George Kotovych

Loren Hepler

Ann English
External Examiner

Date December 13, 1988

This thesis is dedicated to Ian and Olga.

ABSTRACT

The transient and steady state kinetics of the oxidation of isobutyraldehyde by horseradish peroxidase compounds I and II were investigated. The rate constants for the reaction of the enol form of isobutyraldehyde with compounds I and II were $(8 \pm 1) \times 10^6$ M^{-1} s^{-1} and $(1.3 \pm 0.3) \times 10^6$ M^{-1} s^{-1} respectively. The keto-enol tautomerism equilibrium constant was determined to be $(1.7 \pm 0.3) \times 10^{-4}$. The rate constants of the forward and reverse phosphate catalyzed isobutyraldehyde tautomerism reactions were determined by steady state techniques. Rate and equilibrium constants for the tautomerism of the simple linear aldehydes propanal and butanal were also determined through the use of compound I of horseradish peroxidase.

It was shown that the oxidation of malonaldehyde by horseradish peroxidase was due to normal peroxidative enzymology with oxygen consumption resulting from the further reaction of the free radical peroxidation products. Manganese(II) is required for the reaction to take place and its role appears to be in the generation of an endogenous peroxide.

A cyanide binding study on prostaglandin H synthase was carried out. The dissociation equilibrium constant for cyanide and the enzyme was 65 ± 10 μM. The binding rate constant was $(2.8 \pm 0.2) \times 10^3$ M^{-1} s^{-1} at pH 8.0. The pH dependence of the second-order binding rate constant was studied within the pH range of 4.0 - 8.0 and a heme-linked acid dissociation with a pK_a of 4.15 ± 0.10 was discovered with citrate buffer.

A spectral study on the reaction of the enzyme with peroxide and reducing substrates has yielded a method for determining relative reducing substrate ability among compounds. This method involved measuring the rate at which the enzyme emerged from the steady state after oxidation by the peroxide 13-hydroperoxyoctadeca-9,11-dienoic acid. The results on the compounds that

we tested were (from best to worst reducing ability) N,N,N',N'-tetramethyl-*p*-phenylenediamine > phenol > indomethacin > phenylbutazone >> aspirin.

A spectral comparison of the peroxidase and cyclooxygenase reactions by rapid-scan techniques revealed that the enzyme intermediates involved in both reactions are identical. This suggests that the mechanism of the oxidation of arachidonic acid by prostaglandin H synthase may be a peroxidase reaction in which the free radical peroxidation product goes on to react with dissolved molecular oxygen to give the endoperoxide prostaglandin G_2.

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CHAPTER ONE

INTRODUCTION

1.1. PEROXIDASE ENZYMOLOGY

1.1.1. Definition of a Peroxidase

Peroxidase enzymes are oxidoreductases which utilize peroxides to oxidize molecules. In the rules for enzyme nomenclature recommended by the International Union of Biochemistry (IUB), peroxidases fall into the sub-sub-class 1.11.1 (1). Different peroxidases react with different reductants and they are classified further on this basis. For the majority of peroxidases, however, there is a marked lack of specificity with regard to reducing substrate so these classifications must be viewed with caution.

1.1.2. Biological Function

Because of the large number of reactions that can be catalyzed by peroxidases, their biological role has not yet been firmly established. They have been implicated in control of plant growth, lignin degradation and formation, cellular defense, and respiration (2,3). The influence of peroxidases expands considerably when one considers that prostaglandin biosynthesis is a peroxidase dependent process through the enzyme PGH synthase[*] (4), and therefore all of the many roles attributed to the prostaglandins (5-7) are also regulated by a peroxidase.

---

[*] Abbreviations: PGH synthase, prostaglandin H synthase (EC 1.14.99.1); HRP, horseradish peroxidase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; HRP-I, compound I of horseradish peroxidase; HRP-II, compound II of horseradish peroxidase; NMR, nuclear magnetic resonance; HRP-III, compound III of horseradish peroxidase; DDC, diethyldithiocarbamate.

1.1.3. Sources and Some Examples of Peroxidases

Peroxidase enzymes are found in a wide variety of cells in microorganisms, plants, and animals. Peroxidases are typically metalloenzymes and most have heme as the prosthetic group (Figure 1.1). Some notable exceptions include NADH peroxidase, which is a flavoenzyme (8,9) and glutathione peroxidase that has one atom of selenium per subunit of enzyme (10,11).

Peroxidase activity was first discovered in horseradish in 1903 (12) and since then peroxidase content has been found in many other plants (3) with HRP and turnip peroxidase being the best known.

Microorganisms yield a wide variety of peroxidases. Cytochrome c peroxidase from aerobically grown yeast is a very well studied enzyme, and it is the only peroxidase for which the X-ray crystal structure has been determined (13,14). However lignin peroxidase, an extracellular enzyme from the white rot fungus *Phanerochaete chrysosporium* (15,16) has recently been crystallized (17). The group that has done this crystallization is now working toward determining the enzyme structure by X-ray crystal analysis (17). Another extracellular enzyme produced from this white rot fungus is manganese peroxidase (18-21). It mediates its effect by the oxidation of manganese(II) to manganese(III) which, in turn, oxidizes a variety of dyes, phenols, and amines (18-21). Chloroperoxidase (22-24) and bromoperoxidase (25,26) are both halide peroxidases isolated from microorganisms that utilize the indicated halide anion as a preferred reducing substrate.

There are also many animal peroxidases that have been studied extensively. Myeloperoxidase is isolated from neutrophil granulocytes (27-29), and it is believed to play an important role in the body's defense mechanism (30). More recently another white blood cell enzyme, eosinophil peroxidase,

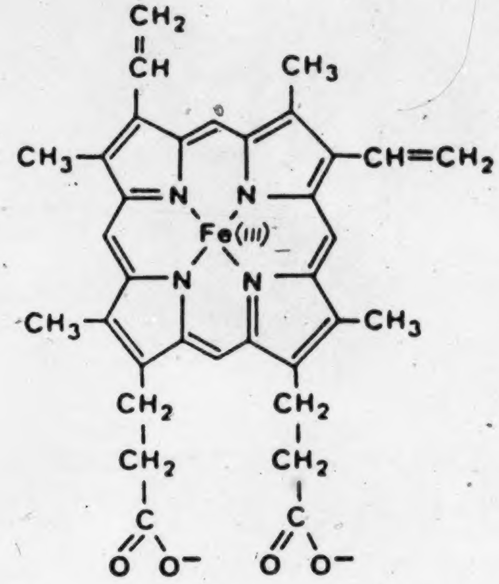


Figure 1.1. Chemical structure of Fe-protoporphyrin IX or heme; the prosthetic group for most peroxidases.

has been characterized (31,32). It is believed to play a part in anti-bacterial defense as well (33). Another animal peroxidase of importance that has been studied extensively is lactoperoxidase from milk (34). The peroxidase prostaglandin H synthase is responsible for the conversion of arachidonic acid to the endoperoxides PGG_2 and PGH_2, which are the precursors to the prostaglandins, thromboxanes, and prostacyclin (7). Finally, the recent isolation and characterization of human salivary peroxidase has been reported (35).

1.2. HORSERADISH PEROXIDASE

1.2.1 Native Enzyme Structure

Horseradish peroxidase is isolated from the root of horseradish where it is found in high concentrations. The enzyme has heme as the prosthetic group. The glycoprotein is a monomer of molecular weight 42kD with 18% carbohydrate (36,37). The enzyme has two calcium ions bound per enzyme molecule (38,39) and it has recently been determined that one of these is necessary for maintenance of a protein structure which is suitable for peroxidase activity (40,41). The heme ferric iron is five-coordinate with the fifth position taken up by the imidazole nitrogen of a histidine residue (42-45). Recent nuclear magnetic resonance work on the protonation state of this proximal histidine shows that it is deprotonated when the iron is in the reduced state (46). In the ferric form the spin state of the iron has been considered to be high spin (47-49). However EPR studies led to a proposal that HRP is actually in a quantum mixed-spin state (50,51). The sixth coordination position of the iron has been thoroughly demonstrated to be unoccupied (52-55). The primary structure of HRP has been determined (36,37). There are 308 amino acid residues, including four disulfide bridges, in a single polypeptide chain that has eight neutral carbohydrate side chains.

The crystal structure of HRP has not yet been determined as it has for cytochrome c peroxidase (13,14). Therefore the active site structure must be elucidated by other techniques. These include spectroscopic techniques as well as sequence homologies to cytochrome c peroxidase (13,14,56). A computer modelling study of the amino acid sequence of HRP shows similarities to cytochrome c peroxidase in peptide structure (56). In particular, the α-helices predicted for HRP follow that observed in the X-ray crystal structure of cytochrome c peroxidase, the hydrophobic/hydrophilic profile predicted for HRP matches that observed for cytochrome c peroxidase, the half cysteines match up when fitted to the cytochrome c peroxidase structure, and the two-domain structure of HRP predicted from limited proteolysis experiments is observed in cytochrome c peroxidase (56). When the sequences of HRP and cytochrome c peroxidase are aligned only 18% of the residues are identical; however, there are important sequence segments that are highly conserved (where highly conserved is defined as 8 identities out of 10 consecutive residues). It is believed that these conserved sequences participate in catalysis, heme attachment, and essential peptide-peptide contacts (56). Based on the crystal structure of cytochrome c peroxidase (13,14) and the computer modelling of the amino acid sequence of HRP (56) it is believed that the key amino acid residues responsible for control of activity in HRP are the axial histidine-170 on the proximal side, the distal arginine-38 and the distal histidine-42. Secondary amino acids include separate carboxylate groups that are adjacent to the imidazole rings of the histidine residues and partake in H-bonding (Figure 1.2).

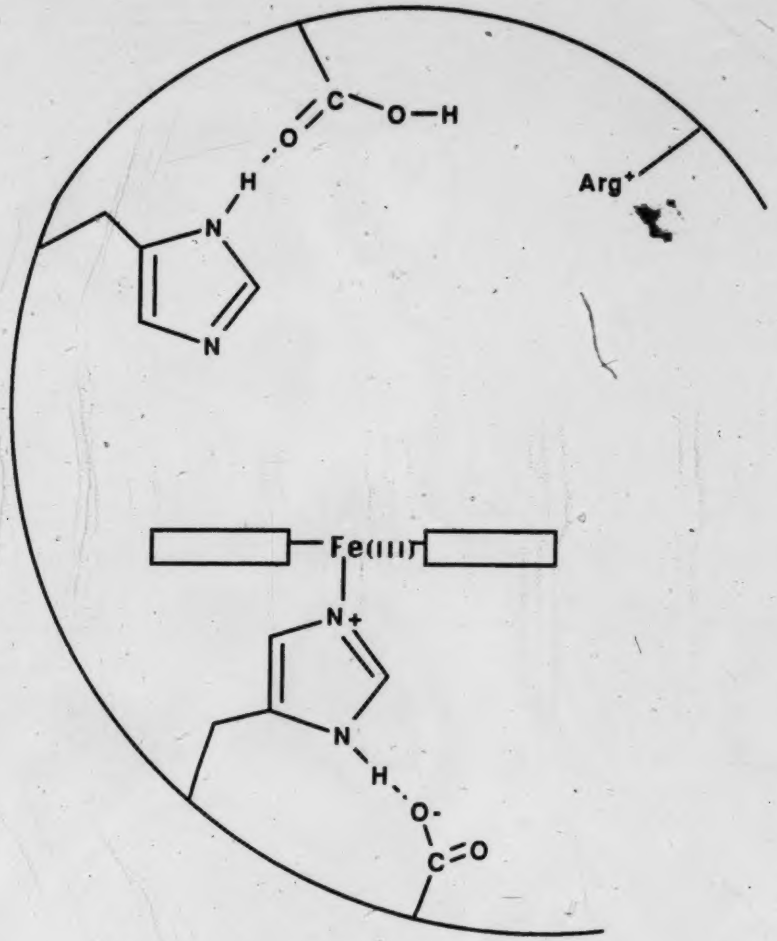

C
O—H
O
H
N
N
Arg+
Fe(III)
N+
N
H
O-
C=O

Figure 1.2. Active site of horseradish peroxidase based on crystal structure of cytochrome c peroxidase (13,14) and computer modelling of the amino acid sequence of HRP.

1.2.2. Mechanism and Enzyme Intermediates

The basic mechanism of reactivity of HRP was proposed in 1952 by Chance (57) and George (58).

[1.1] $HRP + H_2O_2 \rightarrow HRP\text{-}I$

[1.2] $HRP\text{-}I + AH_2 \rightarrow HRP\text{-}II + \bullet AH$

[1.3] $HRP\text{-}II + AH_2 \rightarrow HRP + \bullet AH$

The first reaction is the oxidation of the enzyme by hydrogen peroxide to the enzyme intermediate compound I. Reaction of a reducing substrate with compound I leads to free radical formation and the formation of compound II. This enzyme intermediate can also oxidize a reducing substrate to a free radical and the enzyme returns to the native state. It is generally accepted that the structure of the intermediates is independent of the oxidizing and reducing substrates that are used to form them.

The reaction of peroxide with HRP yields an iron-oxygen structure as indicated by peroxide titration experiments (59). The iron of compound I is blocked to cyanide binding (60). No free protons are gained or lost to the enzyme or solution in the compound I forming reaction (59,61). Therefore the reaction is:

[1.4] $Fe(III) + H_2O_2 \rightarrow (R^{\bullet +})Fe(IV)\text{-}O + H_2O$.

Compound I is represented by $(R^{\bullet +})Fe(IV)\text{-}O$ with a ferryl-type structure. Kinetic studies reveal a catalytically important heme-linked acid dissociation with a pK_a value ≤ 3, which is believed to be due to aspartate-43 (62). Compound I is formally two oxidizing equivalents above that of the native state with an oxidation number of +5. Mossbauer experiments show that the iron is best represented with the iron in the +4 state (63) with the other oxidizing equivalent somewhere else in the active site. Model compound studies have shown (64) and double resonance studies (65), NMR (66), theoretical calculations (67,68), and resonance Raman spectroscopy (69) have verified that the other oxidizing equivalent is in the form of a π cation radical delocalized on the surrounding porphyrin ring. However, just when the question of the location of the other oxidizing equivalent appeared to be settled, it was reported in a paper by LaMar that the cation delocalization is not limited solely to the porphyrin ring. There is apparently significant delocalization to the proximal histidine-170 (70). Kinetic experiments on the reactions of HRP-I with various reducing substrates show that there is a catalytically important heme-linked acid dissociation with a pK_a of between 5.1 and 5.4 (71-74). The same pK_a of 5.4 has been detected on HRP-I by spectrophotometric titration and proton balance (75). The amino acid residue responsible for this pK_a appears to be histidine-42 on the distal side of the heme (76).

The iron in compound II is also on the +4 oxidation state but there is no porphyrin π cation radical. Kinetic and resonance Raman experiments with compound II of HRP show that there is a catalytically important heme-linked acid dissociation with a pK_a of 8.6 (77-80).

There is another form of the enzyme of catalytic importance, compound III. The formal oxidation state of compound III is +6. It can be formed by a number of methods. The addition of superoxide anion to the native enzyme (81), reduction of the enzyme to the ferrous state followed by addition of molecular oxygen (82,83), the reaction of H_2O_2 with HRP-II (84,85), or the reaction of a large excess of H_2O_2 with the native enzyme (86) all yield compound III. There are at least two ways to look at the electronic structure of HRP-III; oxygen-ferroperoxidase or superoxide-ferriperoxidase. HRP-III does not participate in the normal peroxidative cycle; however, it does react very quickly with the plant hormone indoleacetic acid, suggesting that this reaction may be important in plant physiology (87-94).

The optical spectral properties of native HRP were first measured by Kuhn *et al.* in 1931 (95) and based on the similarity of the spectrum obtained with that of ferroporphyrins it was concluded that HRP contains hematin. The optical spectral properties of native peroxidases and the enzyme intermediates have been extensively reviewed (77).

1.2.3. Practical Uses for Horseradish Peroxidase

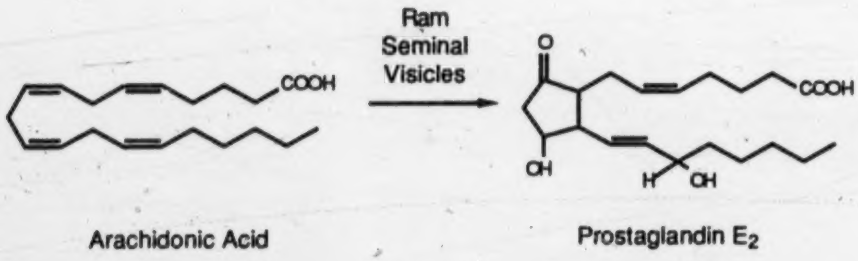
Horseradish peroxidase has become a chemical commodity of value. It has been used for removal of hazardous pollutants from industrial aqueous effluents (96). This method of waste removal involves the treatment of aqueous solutions containing pollutants with hydrogen peroxide and HRP. The enzyme oxidizes the phenolic compounds and aromatic amine waste to free radicals which polymerize and precipitate from the water. Preliminary tests indicate that this process is economically feasible (96). It is also used in enzyme electrodes. One example is in the determination of isocitrate where HRP is one half of a bienzyme electrode. The other enzyme is D-isocitrate dehydrogenase which is co-immobilized with HRP in gelatin and coupled to a Clark-type oxygen electrode (97). Another interesting application of HRP is the photovoltaic determination of H_2O_2 by a biophotodiode (98). This device uses the light emitting reaction of luminol and H_2O_2 catalyzed by immobilized HRP. The light emitted is detected by a photodiode in the electrode adjacent to the immobilized enzyme. The emission is directly proportional to the H_2O_2 concentration.

1.3. PROSTAGLANDIN H SYNTHASE

1.3.1. Biological Function

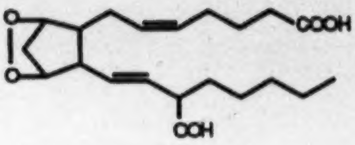
The structure of prostaglandins was elucidated in the early 1960's (99-102). The link between the fatty acids and prostaglandins was made in 1964 (103,104) when it was discovered that an enzyme system present in the seminal vesicle glands of sheep catalyzes the conversion of certain unsaturated fatty acids into prostaglandins.

[1.5]

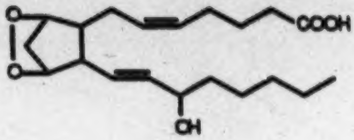


In 1965 oxygen labeling experiments by Samuelsson showed that the oxygen atoms on carbons 9 and 11 of prostaglandin E_1 were from the same molecule of molecular oxygen (105). This led to the postulation of an endoperoxide intermediate in the conversion of unsaturated fatty acids to prostaglandins (105). An endoperoxide was isolated in 1973 (106) and the next year a second endoperoxide was reported (107). These were called PGH_2 and PGG_2. The two intermediates are very unstable in aqueous solution.

[1.6]



PGG$_2$



PGH$_2$

It was postulated (107) that PGG$_2$ is the first stable compound formed from arachidonic acid by the enzymatic activity in ram seminal vesicles. Isolation of the pure enzyme was hampered by the fact that the enzyme is membrane-bound (108). As a general rule, membrane-bound enzymes are more difficult to purify than lipid-free proteins. In the early to mid-70s the techniques of solubilization advanced to the point where the enzyme could be isolated. In 1974 PGH synthase was isolated with low purity from the crude microsomal extract through the use of the non-ionic detergent Tween 20 (109). In 1976 the enzyme was isolated in pure form from ram (4,110) and bovine (111) seminal vesicles. At that time it was realized that the enzyme was responsible for both the cyclooxygenation of arachidonic acid to the hydroperoxy endoperoxide PGG$_2$ and the peroxidase-like reaction converting PGG$_2$ to PGH$_2$ (4,112). The enzyme has been called cyclooxygenase, prostaglandin synthetase, and prostaglandin endoperoxide synthetase; however the name commonly accepted now is prostaglandin H synthase (EC 1.14.99.1); and that name will be used throughout this thesis.

1.3.2. Structure and Properties

PGH synthase is a heme-containing enzyme (110,111) with an absorbance peak in the Soret region at 410 nm. The enzyme exhibits only one protein band upon SDS gel electrophoresis with a molecular weight of 70 kD (4,110, appendix A.2). Crosslinking and ultracentrifugation experiments show that PGH synthase is a dimer (4,112). The amino acid sequences have been determined from gene sequencing studies and the enzyme has 599 (113) or 600 (114) amino acids. The enzyme's location on the endoplasmic reticulum (115) with the activity oriented toward the cytoplasm as opposed to the luminal side(116) has been identified by immunocytochemistry.

The stoichiometry of heme:enzyme dimer has been the subject of several papers with the results varying from 4:1 (1197), 2:1 (118), and 1:1 (119). Because the enzyme does contain heme it has a strong absorbance in the Soret region (117). The spectral changes associated with the enzyme reaction with 5-phenylpent-4-enyl-1-hydroperoxide (120) and PGG_2 (121) have been studied and show similar behavior to that observed for the intermediate compounds of HRP.

1.3.3. Prostaglandin H Synthase Reactions

The reaction of arachidonic acid with PGH synthase is oxygen consuming and can therefore be followed through the use of a Clark-type polarographic oxygen electrode. This monitoring of enzyme activity is called the cyclooxygenase assay and was first used on crude enzyme preparations in 1972 (122). The assay involves the addition of arachidonic acid to the enzyme preparation followed by monitoring of the oxygen consumption. It was noted that phenol has a stimulatory effect on oxygen consumption (122) and so in most assay procedures described in the literature 0.5-1.0 mM phenol is added to the reaction mixture to maximize the observed signal. With preparations that are not 100% holoenzyme, hematin is added in excess to reconstitute the enzyme. A typical set of cyclooxygenase assay conditions is 200 µM arachidonic acid, 0.2 µM prostaglandin H synthase, 0.5 mM phenol, and 1 µM hematin at 30°C, phosphate buffer, pH 8.0. It was observed that two moles of oxygen were consumed per mole of arachidonic acid oxidized (122). A unit of enzyme is defined as the amount of enzyme that will oxidize one μmole of arachidonic acid per minute. It was noted as early as 1972 that the cyclooxygenase activity leads to irreversible inactivation of the enzyme (122). This may be a self-regulating deactivation process. Once the enzyme begins to function, it will produce no more than a fixed, finite amount of endoperoxide to serve as a precursor to the prostaglandin hormones. It is not clear, however, if this inactivation process would occur *in vivo* or not because the enzyme is membrane-bound in the cell (108) and this probably helps to stabilize the enzyme somewhat.

Experiments showed that upon addition of glutathione peroxidase to the cyclooxygenase reaction inhibition of cyclooxygenase activity was observed (123,124). The addition of the peroxide-scavenging glutathione peroxidase would eliminate peroxide. Therefore it was suggested that hydroperoxide plays a role in the arachidonic acid reaction with PGH synthase. Addition of glutathione peroxidase during the reaction also causes cyclooxygenase activity to cease. This indicates that the peroxide requirement is not simply for initiation but is needed continuously (123,124).

By operating under conditions of very high diethyldithiocarbamate concentration (DDC is known to reduce peroxide) it was possible to see a lag in oxygen consumption; and this lag can be eliminated by the addition of certain peroxides (125). PGG_2 and other lipid hydroperoxides were effective in reducing the lag time for cyclooxygenase activity at μM concentrations. The straight chain lipid hydroperoxides are more effective than PGG_2 in triggering rapid oxygenation rates (125). Hydrogen peroxide, t-butyl peroxide, and cumene hydroperoxide had no effect on lag times at the μM level.

EPR work on crude (126) and purified (127) enzymes showed that cyclooxygenase activity involved radical intermediates. Another piece of evidence which suggested radical intermediates was the fact that the cyclooxygenase reaction was strongly inhibited by antioxidants (128,129). A mechanism suggested by Eling (127) for the reaction of arachidonic acid with the enzyme was based on his EPR studies. It involved hydrogen atom abstraction at C_{13} by the enzyme followed by rearrangement to give the radical at C_{11}. Scavenging of the radical by dissolved molecular oxygen gives a peroxyl radical, which partakes in an intramolecular 5-exo cyclization to give the endoperoxide and a radical at C_8. This would cyclize again to give a radical at C_{15}, which would then react with a molecule of dissolved molecular oxygen to form another peroxyl radical, which in turn could abstract a hydrogen atom to form the hydroperoxy endoperoxide PGG_2. One important question must be addressed if this mechanism is to be believed. What is the enzymatic mechanism of hydrogen atom abstraction to give the arachidonic acid radical at C_{13}? One postulation for the mechanism of the enzymatic activity is through a peroxidase-like reaction. This involves the reaction of peroxide with native enzyme to give compound I followed by reaction of arachidonic acid with the enzyme intermediate to give the arachidonic acid radical described previously (120,130-133). There is spectral evidence for the formation of a compound I-like enzyme intermediate in the reaction of peroxides with native enzyme (120,121). Work by Dietz *et al.* has shown that a tyrosyl radical is formed in the reaction of the enzyme with both arachidonic acid and PGG_2 or H_2O_2 and they postulated that it is this tyrosyl radical that abstracts the hydrogen atom from C_{13} of arachidonic acid (132).

1.3.4. Inhibition by Nonsteroidal Anti-Inflammatory Drugs

One very interesting aspect of PGH synthase enzymology is the interaction of the enzyme with nonsteroidal anti-inflammatory drugs. The most studied and best understood interaction is with aspirin.

In 1971, Vane reported that aspirin inhibited prostaglandin biosynthesis by inhibition of the enzymatic activity isolated from guinea-pig cell-free lung homogenate (134). Work by Samuelsson's group in 1974 demonstrated that aspirin was inhibiting the conversion of arachidonic acid to the endoperoxides (135,107) and thus was a PGH synthase inhibitor. In 1975, Roth *et al.* presented evidence suggesting that the mechanism of action of inhibition of PGH synthase by aspirin was by acetylation of a serine residue (136,137). Aspirin at a concentration of 100 µM inhibits PGH synthase in 60 minutes (136,137).

One of the consequences of acetylation of the enzyme was that aspirin decreases platelet aggregation. By blocking the production of endoperoxides, the concentration of the hormone responsible for platelet aggregation, prostaglandin E_2, is reduced. This mechanism of reduction of platelet aggregation by inhibition of PGH synthase is believed to explain the therapeutic effect of aspirin on heart patients (138).

1.3.5. Oxidation of Xenobiotics by Prostaglandin H Synthase

The metabolism of xenobiotics often involves oxidation of the molecule converting it into a much more toxic, mutagenic, and carcinogenic species (139). It is slowly being realized that PGH synthase is guilty of at least some of this activation. Two examples of work in the area of xenobiotic oxidation by PGH synthase to yield toxic species are discussed below.

In 1978 Marnett *et al.* reported that 7,8-dihydroxybenzo(a)pyrene was oxidized by PGH synthase to mutagenic derivatives which were determined to be the corresponding epoxide (140). Similar work by Reed *et al.* on the oxidation of the nonsteroidal anti-inflammatory drug phenylbutazone (141) shows that the peroxidase activity of PGH synthase resulted in hydrogen atom abstraction followed by addition of molecular oxygen to yield a peroxyl radical. This appears to be an epoxidizing agent for 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene. The interaction of phenylbutazone with PGH synthase may represent a mechanism for toxicity rather than a therapeutic use because it serves to amplify oxidative capacity rather than reduce it.

Another example of oxidation by PGH synthase is the oxidation of benzidine. Benzidine is a human bladder carcinogen. Metabolic activation by PGH synthase in the bladder could be important. It has been demonstrated that benzidine is oxidized by the enzyme yielding products which bind to DNA (142-144). Oxidation of benzidine by PGH synthase/arachidonic acid and by HRP/H_2O_2 yield the same one-electron oxidized intermediate as detected by EPR techniques and product analysis (145). This intermediate is believed to be the benzidine cation radical.

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CHAPTER TWO

TRANSIENT STATE KINETICS OF THE REACTIONS OF ISOBUTYRALDEHYDE WITH COMPOUNDS I AND II OF HORSERADISH PEROXIDASE[*]

2.1. INTRODUCTION

Horseradish peroxidase can act upon several substrates as an oxidase. Some of these, like indoleacetic acid (1-6), IBAL (7,8),[**] linear aldehydes (9,10) and phenylacetic aldehyde (11-13) generate products in an electronically excited triplet state. In the case of indoleacetic acid and IBAL direct chemiluminescence can be observed. This work is concerned with the IBAL/O_2/HRP system which generates acetone in the triplet state and formic acid. Previously a mechanism for this reaction was proposed and qualitative aspects were described (14,15). In this work it is established that it is only the unionized enol form of the aldehyde which reacts, and the elementary rate constants for the reaction of the enol form of the aldehyde with both HRP-I and HRP-II have been determined. The values of the keto-enol equilibrium constant and the enolization and ketonization rates in the presence of phosphate also have been measured. The enol form of aldehydes appears to react similarly to unionized phenols with compounds I and II of HRP and a common mechanism is proposed.

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[*] A version of this chapter has been published. Bohne, C., MacDonald, I.D., and Dunford, H.B. (1987) *J. Biol. Chem.* **262**, 3572-3578.

[**] Abbreviations: IBAL, isobutyraldehyde; HRP, native horseradish peroxidase (EC 1.11.1.7) donor H_2O_2 oxidoreductase; HRP-I, compound I; HRP-II, compound II; keto, keto form of IBAL; enol, enol form of IBAL; hydrate, hydrated IBAL; [IBAL], total concentration of aldehyde

A first-order dependence of the reaction rate with phosphate was described in earlier work and it was proposed that phosphate plays a role in the enolization. Indeed enol phosphate derivatives from IBAL are more efficient catalysts of enolization than phosphate but are not substrates for the enzyme (16). In this work it is shown that phosphate catalyzes the tautomerism; it does not affect the keto-enol equilibrium constant, nor does it play any other role in the reaction mechanism.

2.2. MATERIALS AND METHODS

The reagents were purchased from the following sources: K_2HPO_4, KH_2PO_4 (both Pro Analysis), HCl, KCl, K_2SO_4 (all Suprapur) from Merck, tris-(hydroxymethyl)methylamine (Aristar) from BDH, $Na_4P_2O_7$, $K_4Fe(CN)_6$ (ACS) from Fisher, NADH (reduced form, grade III), phenazine methosulphate from Sigma, H_2O_2 from Mallinckrodt, ethylenediaminetetraacetic acid (EDTA) from American Chemicals, NaOH (standard solution) from Fluka, horseradish peroxidase (grade I) from Boehringer Mannheim, catalase from Worthington Biochemical, IBAL from Aldrich.

IBAL was distilled under N_2 through a Vigreux column. Stock solutions of various concentrations (below 2.2 M) of IBAL in ethanol were prepared daily. The ethanol is necessary to solubilize the aldehyde in the aqueous reaction mixture. HRP obtained as an ammonium sulphate precipitate was dialyzed for 12 hours at 4°C against a 200:1 excess of deionized water; the water was changed every 3 hours. All stock solutions of HRP had a purity number (A_{403nm}/A_{280nm}) greater than 3.3.

The concentration of HRP (ε_{403nm} of 1.02×10^5 M^{-1} cm^{-1}) (18) were determined spectrophotometrically. H_2O_2 concentration was determined by the peroxidase assay (19). Catalase was used as purchased (60,000 U/mL). HCl concentration was determined by titration with a standard NaOH solution. The dissolved oxygen concentration under our experimental conditions was determined as described elsewhere (20). All other solute concentrations were determined by weight. All solutions were prepared in water purified by a Milli-Q Water Purification System from Millipore. Resistivity of water was at least 10 megaohm cm.

Optical spectra and slow reactions were recorded on a Cary 219 spectrophotometer. The stopped-flow measurements were made using a Photal Rapid Reaction Analyzer Model RA-601 equipped with a 1 cm observation cell. Rate constants for experiments performed on this apparatus were determined by non-linear least squares curve fitting analysis, carried out by computer. Oxygen consumption was followed with a Yellow Springs Instrument Model 53 Oxygen Monitor and pH measurements were performed using a Fisher Accumet Model 420 digital pH meter.

The concentrations of ethanol never exceeded 0.6 M compared to ~55 M H_2O. No change in the pH reading was noted upon ethanol addition.

All reactions were studied at 35°C, ionic strength 0.67 M and pH 7.4. Enzyme concentrations were typically 1.0 μM. The solutions were allowed to sit for 7 minutes in the thermostated chambers of the Rapid Reaction Analyzer prior to the reaction. For the measurements on the Cary spectrophotometer the solutions were kept at 35°C in a separate bath before mixing. The pH was maintained by a KH_2PO_4/K_2HPO_4 buffer. Ionic strength was kept constant by addition of KCl or K_2SO_4. No difference was observed in the results when these salts were interchanged.

Compound I of HRP was prepared by the addition of a slightly lower than stoichiometric amount of H_2O_2 to native enzyme (21). The spectrum of compound I was monitored repeatedly each day to confirm stability. The stability decreases at higher enzyme concentrations.

A high yield of compound II cannot be obtained at pH 7.4, so it was prepared in 2.5 mM tris(hydroxymethyl)methylamine/HCl buffer at pH 9.0 and a pH jump method was used. First compound I was made and then it was reduced to compound II by the addition of a stoichiometric amount of $Fe(CN)_6^{4-}$ (21). This was placed in one reservoir of the stopped-flow apparatus. A higher concentration of phosphate buffer at pH 7.4 was added to the IBAL solution which was placed in the other reservoir. The final pH of the reaction mixture was 7.4. The inert salt (K_2SO_4) necessary to maintain the ionic strength was added to the compound II solution.

In the equilibrium constant determination the single beam spectrophotometer of the Rapid Reaction Analyzer was calibrated by the use of HRP solutions with known absorbance values. The concentration of compound I used in the determinations of K_{enol} was 5.0 μM.

The chemiluminescent reaction was observed in a dark room under the following conditions: a total volume of 30 mL of solution was used which contained 0.6 M phosphate buffer at pH 7.4, 73.3 mM IBAL, 85 μM H_2O_2, and 1.0 μM HRP. The reaction mixture was thermostated at 40°C prior to the reaction. Five minutes were allowed to elapse before the reaction was initiated so that the eyes could adapt to the dark. The flask was then removed from the constant temperature bath and IBAL followed by HRP was added while stirring.

2.3. RESULTS

Our results on the elementary reactions of compounds I and II with isobutyraldehyde can be accounted for quantitatively with the following equations:

[2.1]
$$\begin{tikzcd} \text{keto + phosphate} \arrow[r, shift left, "k_1"] \arrow[d, shift left, "H_2O"] & \text{enol + phosphate} \arrow[l, shift left, "k_{-1}"] \\ \text{hydrate} \arrow[u, shift left, "H_2O", "K_{hyd}"'] & \end{tikzcd}$$

[2.2]
$$K_{hyd} = \frac{[\text{hydrate}]}{[\text{keto}]}; \quad K_{enol} = \frac{[\text{enol}]}{[\text{keto}]} = \frac{k_1}{k_{-1}}$$

[2.3]
$$\text{HRP-I + enol} \xrightarrow{k_{3app}} \text{HRP-II + radical}$$

[2.4]
$$\text{HRP-II + enol} \xrightarrow{k_{4app}} \text{HRP + radical}$$

For resonance structures of the radical and a proposed detailed mechanism of the overall reaction see Appendix B. The rate of reaction of compound I is given by:

[2.5]
$$-\frac{d[\text{HRP-I}]}{dt} = k_{3app}[\text{enol}][\text{HRP-I}] \ .$$

With [enol] >>[HRP-I] pseudo-first order conditions are observed with the rate constant k_{3obs} given by:

[2.6]
$$k_{3obs} = k_{3app}[\text{enol}] \ .$$

In aqueous solution IBAL exists in the three forms indicated in Equation [2.1]. The enol concentration is low. Therefore the total aldehyde concentration is given by:

[2.7] $$[\mathrm{IBAL}] \cong [\mathrm{keto}] + [\mathrm{hydrate}] \ .$$

Combination of Equations [2.2] and [2.7] leads to:

[2.8] $$[\mathrm{enol}] = \frac{K_{enol}[\mathrm{IBAL}]}{1 + K_{hyd}} \ .$$

Substitution into Equation [2.6] yields

[2.9] $$k_{3obs} = \frac{k_{3app}K_{enol}[\mathrm{IBAL}]}{1 + K_{hyd}} \ .$$

The reaction between compound I and the enol form of IBAL (Equation [2.3]) was followed at 411 nm, the isosbestic point between the native enzyme and compound II. The reaction is first order and as predicted by Equation [2.9], the dependence of k_{3obs} upon [IBAL] is linear (Figure 2.1). The inset shows the absorbance change with time at 411 nm. From the slope of the linear plot in Figure 2.1, k_{3app} can be calculated if K_{hyd} and K_{enol} are known. We use a value for K_{hyd} of 0.45 at 35°C, obtained from the literature (22,23) and the determination of K_{enol} is described below. The mean value of k_{3app} is listed in Table 2.1.

Analogous equations are obtained for the rate of the reaction between compound II and the enol:

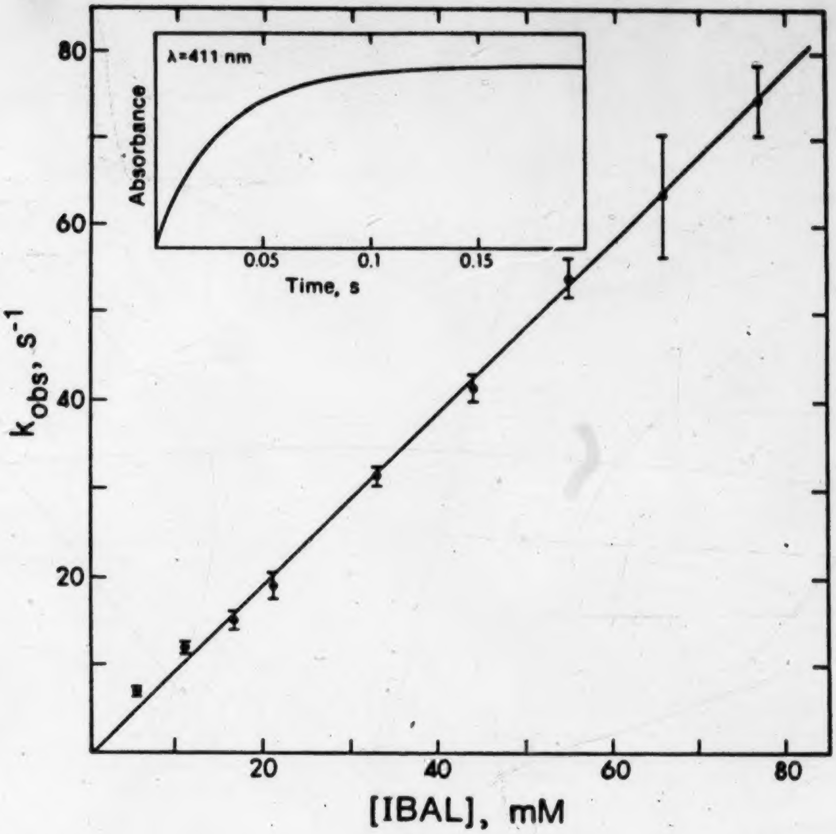

Figure 2.1. Plot of k_{obs} *versus* [IBAL] for the reaction between the enol of IBAL and compound I in phosphate buffer 0.1 M, pH 7.4, KCl 0.422 M (μ = 0.67 M), compound I 1.0 μM and ethanol (0.47-0.58 M). The inset shows a typical trace at 411 nm. The mean value for the slope is $(9.4 \pm 0.5) \times 10^2$ $M^{-1}s^{-1}$.

Table 2.1. Rate Constants for Various Elementary Reactions and Equilibrium Constants Measured at 35°C, pH 7.4, Ionic Strength 0.67M in Dilute Aqueous Ethanol.

K_{enol} [a]	$(1.7 \pm 0.3) \times 10^{-4}$
k_1 [b]	$(8.5 \pm 0.4) \times 10^{-5}$ M^{-1} s^{-1}
	$(8.9 \pm 0.4) \times 10^{-5}$ M^{-1} s^{-1}
k_{-1} [c]	$(5 \pm 1) \times 10^{-1}$ M^{-1} s^{-1}
k_2 [d]	1×10^{7} M^{-1} s^{-1}
k_{3app} [e]	$(8 \pm 1) \times 10^{6}$ M^{-1} s^{-1}
k_{4app} [f]	$(1.3 \pm 0.3) \times 10^{6}$ M^{-1} s^{-1}

(a) [enol]/[keto] equilibrium constant corrected for hydrate formation.

(b) enolization rate constant in the presence of phosphate determined both from the reaction with HRP-I and from the overall reaction monitored by rate of O_2 consumption.

(c) ketonization rate constant in the presence of phosphate.

(d) rate constant for the reaction between HRP(native) and peracid estimated from related literature values (25,26).

(e) rate constant for the reaction between compound I and the enol form of IBAL.

(f) rate constant for the reaction between compound II and the enol form of IBAL.

[2.10] $$-\frac{d[\text{HRP-II}]}{dt} = k_{4app}[\text{enol}][\text{HRP-II}]$$

and when [enol] >> [HRP-II]

[2.11] $$k_{4obs} = \frac{k_{4app}K_{enol}[\text{IBAL}]}{1 + K_{hyd}}$$

This reaction was studied at 425 nm, the isosbestic point between native HRP and compound I. The linear relationship between k_{4obs} and [IBAL] is shown in Figure 2.2. The value of k_{4app} is listed in Table 2.1.

The reaction between compound II and the enol is the slowest reaction in the enzymatic cycle. The autoxidation product of IBAL is the peracid and is always present (15). Therefore a recycling of the enzyme occurs as soon as HRP is formed in the reaction between compound II and the enol. In the initial stages of the compound II reaction first-order kinetics is observed, but at longer times recycling takes place (inset Figure 2.2). The recycling is more prominent at higher IBAL concentrations because the peracid concentration is high relative to the enzyme concentration.

The amounts of enol initially present at three different IBAL concentrations were determined when [HRP-I]>[enol]. Under these conditions a rapid disappearance of compound I due to the enol initially present was observed prior to the onset of a slower reaction of compound I (Figure 2.3). Thus there is a burst followed by a steady state reaction. The rate limiting step of the slower reaction is the enolization. The single beam Rapid Reaction Analyzer was calibrated so that the final absorbance value for the rapid initial reaction could be obtained. The initial absorbance value and the molar absorptivity for compound I at 411 nm were measured on the Cary spectrophotometer. The

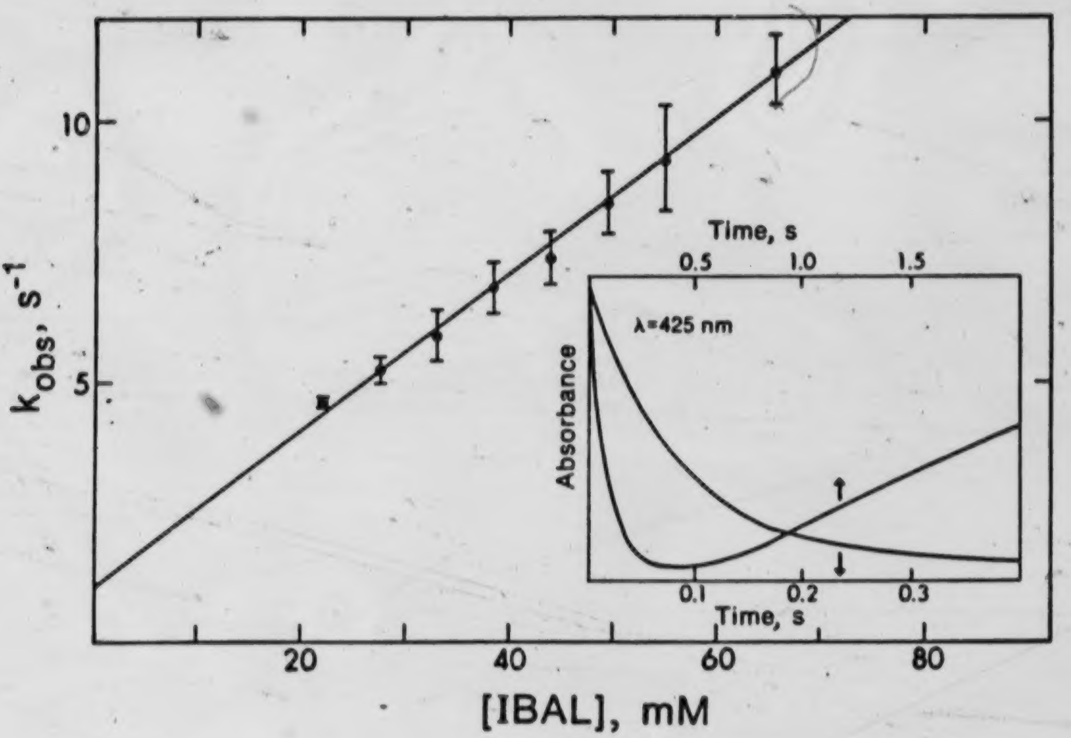

Figure 2.2. Plot of k_{obs} *versus* [IBAL] for the reaction between the enol of IBAL and compound II in phosphate buffer 0.1 M pH 7.4, Tris 1.25 mM, K_2SO_4 0.147 M ($\mu = 0.67$ M), compound II 1.0 μM and ethanol 0.49-0.57 M. The inset shows typical reaction traces at 425 nm at two different time scales. The mean value for the slope is $(1.53 \pm 0.06) \times 10^2\ M^{-1}\ s^{-1}$.

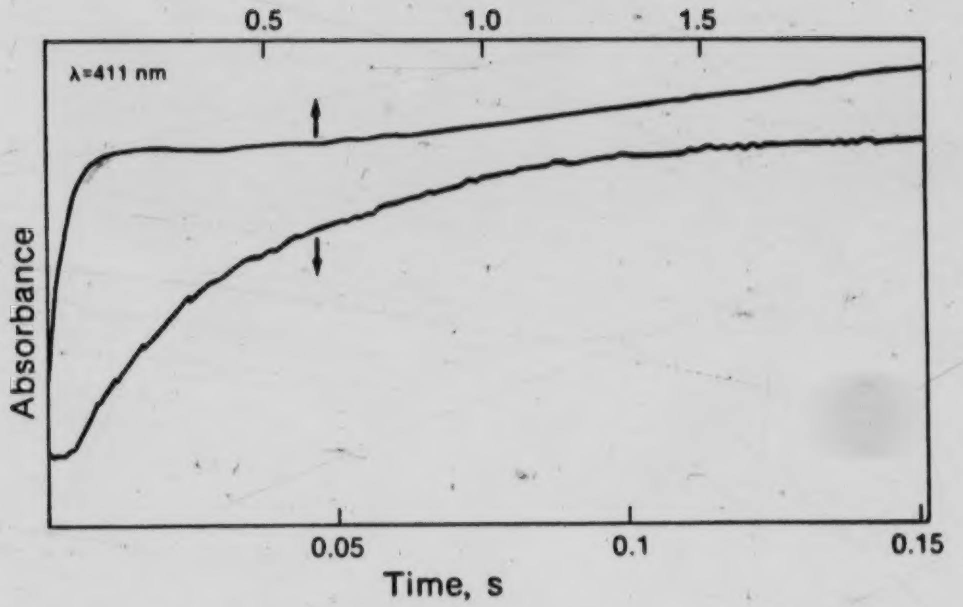

Figure 2.3. Reaction traces at 411 nm on two different time scales under conditions of [enol] << [HRP-I], for the reaction of enol with HRP-I. Phosphate buffer 0.1 M, pH 7.4, KCl 0.422 M ($\mu = 0.67$ M), compound I 5.0 μM, IBAL 9.63 mM and ethanol 0.118 M.

change in absorbance observed for the fast burst was related to the compound I concentration change through Beer's law. Thus the equilibrium concentrations of enol were obtained and K_{enol} was readily calculated. As compound I is not very stable at 5.0 µM, a blank experiment was run on the Cary spectrophotometer monitoring the increase in absorbance with time. For each reaction in the Rapid Reaction Analyzer the spontaneous decomposition of compound I was taken into account. The value for the equilibrium constant between enol and keto, K_{enol}, was obtained for three different IBAL concentrations (9.63 mM, 14.58 mM and 19.25 mM) in two independent sets of experiments. The mean value is:

$$K_{enol} = (1.7 \pm 0.3) \times 10^{-4};\ pK_{enol} = 3.77 \pm 0.08$$

Phosphate has no effect on the keto-enol equilibrium constant. The value of K_{enol} obtained in this study is similar to that published (24) for the equilibrium constant in water at 25°C ($K = 1.37 \times 10^{-4}$; $pK = 3.86 \pm 0.03$).

The effect of phosphate concentration on k_3 and k_4 was studied in the range of 0.01 M to 0.3 M phosphate for compound I and 0.075 M to 0.3 M for compound II. It was shown in previous work that the overall reaction depends on phosphate in this concentration range (16). No effect of phosphate was observed on these rate constants. In the experiments with compound II it was not possible to work at lower phosphate concentrations. A certain excess of phosphate was required to obtain a final pH of 7.4, as compound II was prepared in Tris pH 9.0 buffer.

In earlier work EDTA or pyrophosphate was added to enhance the emission of triplet acetone. These substances chelate metal ions which, by quenching the excited species or perhaps by inducing dark decomposition of the dioxetane intermediate, diminish the emission intensity (8). We could not use pyrophosphate in the present studies as all available samples contained impurities which reacted with the intermediate enzyme compounds. When EDTA (1.0×10^{-4} M) was added to the reaction mixture, no change was observed for the value of the rate constant for the reaction between compound I and the enol. As compound I was slightly unstable in the presence of EDTA, all kinetic experiments in the present study were performed without a chelating agent.

The reaction between compound I and the enol form of IBAL was studied in Tris buffer. The rate of the reaction is smaller in the 0.15 M ($\mu = 0.15$ M) pH 7.4 Tris buffer. With the addition of 0.15 M ($\mu = 0.332$ M) of phosphate buffer (pH 7.4) to the Tris no increase of the rate constant was observed. It would appear therefore that Tris buffer has an inhibiting effect.

When [HRP-I]>[enol], the slow reaction, after the burst in which enol initially present is consumed (Figure 2.3), has an initial zero-order dependence (Figure 2.4 inset). The dependence of the zeroth-order rate constant, $k_{z.o.}$, upon [IBAL] (not shown) or [phosphate] (Figure 2.4) is linear. These experimental results support a rate law in the slow region of:

$$[2.12] \qquad -\frac{d[\text{HRP-I}]}{dt} = \frac{k_1\,[\text{IBAL}][\text{phosphate}]}{1 + K_{hyd}} = k_{z.o.}$$

This expression can be derived using the steady state approximation for [enol] (see Appendix B). Since $k_{z.o.}$, [IBAL], and [phosphate] are all measured experimentally, k_1, the enolization rate constant, is readily calculated (Equation [2.12]). The blank rate of compound I decomposition was always subtracted. The mean value of k_1 is $(8.5 \pm 0.4) \times 10^{-5}\ M^{-1}\ s^{-1}$.

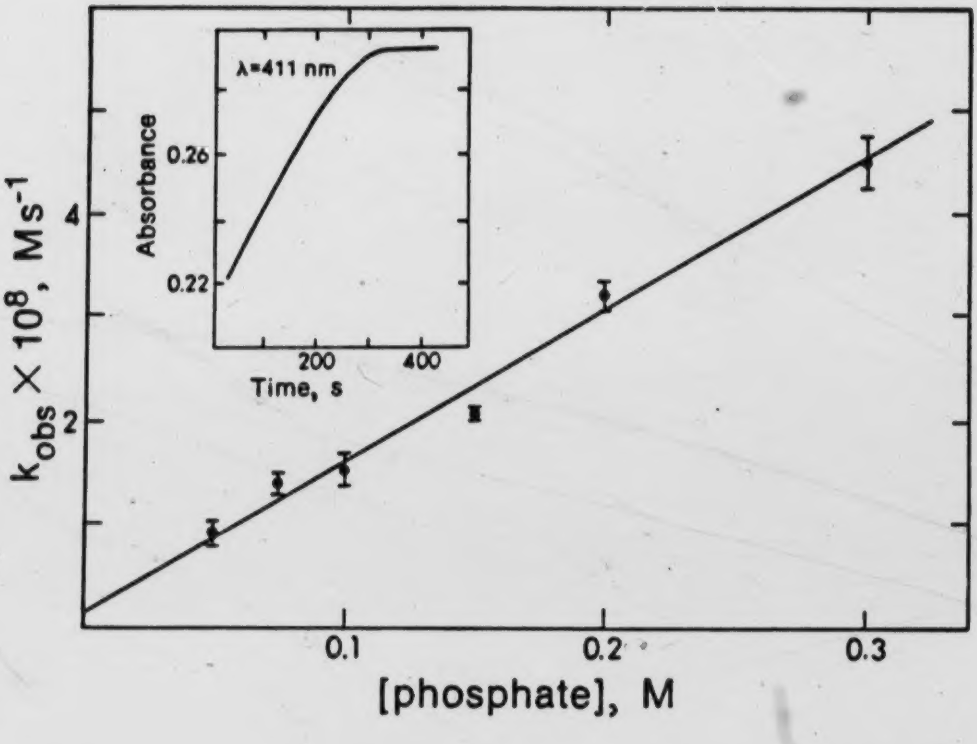

Figure 2.4. Plot of k_{obs} *versus* [phosphate] for the reaction between enol of IBAL and compound I under steady state conditions. Phosphate buffer pH 7.4, KCl (0-0.555 M) (μ = 0.67 M), compound I 5.0 μM, IBAL 2.06 mM and ethanol 0.12 M. The inset shows a typical reaction trace at 411 nm.

The overall reaction can be monitored by observing O_2 consumption (15). We determined the dependence of the rate constant for O_2 consumption upon phosphate concentration at a constant ionic strength of 0.67 M (16). The O_2 concentration was determined to be $(1.08 \pm 0.07) \times 10^{-4}$ M. We confirmed earlier experiments (16) which showed that the dependence of the overall reaction with phosphate is linear (Figure 2.5). Applying the steady state approximation to the overall reaction the following expression is obtained (see Appendix B):

[2.13]
$$-\frac{d[O_2]}{dt} = \frac{k_1[\mathrm{IBAL}][\mathrm{phosphate}]}{1 + K_{hyd}} = k_{z.o.}$$

The value of k_1 $(8.9 \pm 0.4) \times 10^{-5}$ M^{-1} s^{-1} obtained from the overall reaction monitored by oxygen consumption (Equation [2.13]) is in good agreement with that observed in the reaction of the enol with compound I (Equation [2.12]). All results are summarized in Table 2.1.

A very fast light emission with high intensity should be seen in the millisecond time scale, due to the enol initially present. Unfortunately, the available photon-counting system did not cover this short time range (15). Indeed both an initial light flash and the steady state light emission can be seen in a darkroom.

2.4. DISCUSSION

Peroxidases utilize both an oxidizing substrate and a reducing substrate in their normal cycle. The reaction of IBAL and O_2 catalyzed by HRP is no exception. Conversion of native HRP to compound I is caused by the peracid autoxidation product of IBAL or by H_2O_2 added to the reaction mixture (15). The

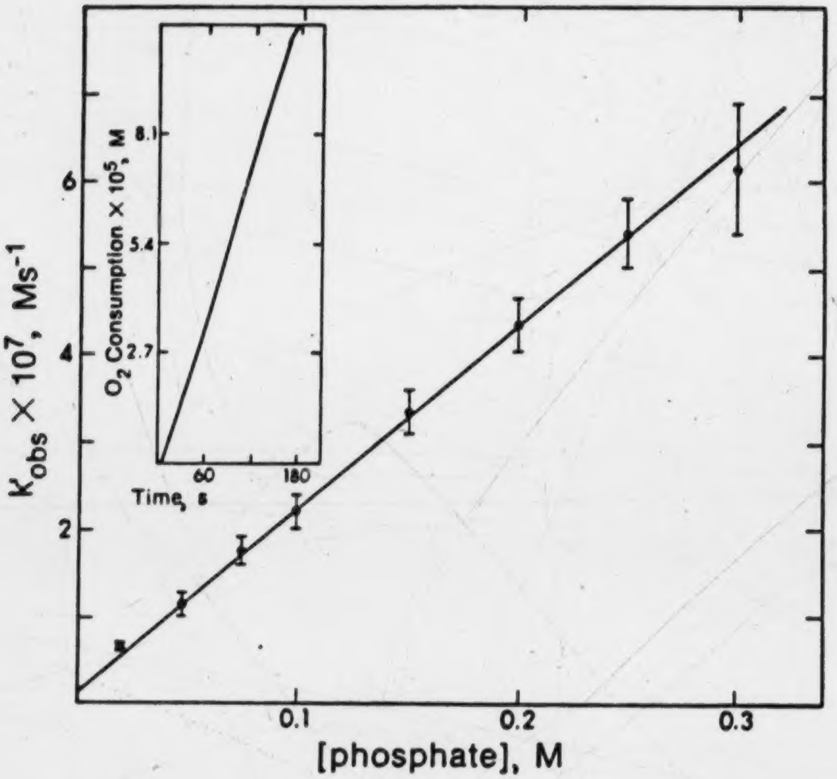

Figure 2.5. Plot of k_{obs} *versus* [phosphate] for the overall reaction monitored by O_2 consumption. Phosphate buffer pH 7.4, KCl (0-0.600)(μ = 0.67 M), HRP 1.0 μM, IBAL 33.0 mM, H_2O_2 6.0×10^{-5} M and ethanol 0.54 M. The inset shows a typical trace for the O_2 consumption experiment.

rate constant for the reaction of the native enzyme with H_2O_2 is 1.8×10^7 $M^{-1}\,s^{-1}$).

The reducing substrate is the enol form of the aldehyde. This has been demonstrated in the present work in a variety of ways. With [compound I] in excess of [enol] an initial burst in compound I disappearance is observed in the millisecond time range, the magnitude of which corresponds to the equilibrium enol concentration. This leads to a prediction of an initial burst in light emission and oxygen consumption which previously had gone undetected because the dead time of the instrument was too long (15). Because of this prediction we performed the qualitative experiment in the darkroom. Human eyes could detect the initial flash of light followed by the lower level of steady state emission. A similar observation was made when the trimethylsilyl enol ether of IBAL was used as a substrate (27). Hydrolysis of the ether in the presence of weak base provided by the buffer or by fluoride ion gives the free enol of the IBAL. When this substrate is added to the reaction mixture followed by enzyme addition a strong light emission is observed that decays rapidly. This is followed by a steady state emission. The intense burst of light is due to a high enol concentration built up as the substrate is added to the reaction mixture.

The steady state disappearance of compound I following the initial burst observed in the present system also indicates enol reactivity. The steady state phase indicates that there is a rate-limiting step prior to the compound I reaction. The rate constant for the rate-limiting step can be measured and it corresponds well with published enolization rate constants (24). Finally the steady state overall reaction rate was followed by monitoring oxygen consumption and within experimental error the same rate constant was measured. Thus the steady state rate of the complex overall reaction is governed by the rate of enolization of IBAL.

The value of the rate constant for the reaction of compound II with enol was also determined (Table 2.1). As is usually observed for HRP, the rate constant for the reaction with compound II is smaller than for the reaction with compound I. Because of this slower reaction for compound II, this compound is observed under steady state conditions (7,15).

For the phosphate dependence study on the reaction between compound I and the enol, the assumption was made that $k_3[\text{HRP-I}] >> k_{-1}$ [phosphate] under steady state conditions. There is a factor of 50 between the two expressions for the highest phosphate concentration. The assumption made for the steady state approximation ($k_3[\text{HRP-I}] + k_4[\text{HRP-II}] >> k_{-1}[\text{phosphate}]$) on the overall reaction also holds. (Under steady state conditions [Enzyme] ≡ [HRP-II].) The value of the ketonization rate constant in the presence of phosphate was obtained from the enolization rate constant and the keto-enol equilibrium constant.

Some important points concerning the mechanism of action of phosphate have been established in this work. The phosphate does not affect the keto-enol equilibrium constant or the rate constants for the reaction between the enol form of IBAL and compound I or compound II. The phosphate does play a catalytic role that acts equally on the enolization and ketonization rate constants. The earlier proposed role of phosphate on the enolization (16) has been confirmed by these results.

The enolate anion cannot be the reducing substrate. The pK_a of the enol is 11.6 (24) so that the concentration of enolate is small at pH 7.4. The experimental value of k_3 is $8.0 \times 10^6\ M^{-1}\ s^{-1}$ and if the enolate were reacting with compound I, not the enol, one obtains a rate constant of $1.3 \times 10^{11}\ M^{-1}\ s^{-1}$. This exceeds the diffusion-controlled limit and therefore is physically impossible (28).

Because the enol, not the enolate anion, reacts with compounds I and II, the aldehydes resemble the phenols, where the unionized phenols, not the phenolate anion, is also reactive with the enzyme (29). This is contrary to what one would expect from the ease of electron donation. However in the case of phenols reacting with horseradish peroxidase there is a large advantage in having the unionized form reactive. If the pK_a of the phenol is 9 then the ratio [phenol]/[phenolate] is 100 at pH 7 and 10^4 at pH 5. Based on the available experimental evidence we offer a common reaction mechanism for phenols and enols reacting with compounds I and II in Figures 2.6 and 2.7. In these figures we describe the reducing substrate as C-O-H, which represents either unionized enol or phenol.

Reaction of enol with compound I involves proton transfer to a distal base B and electron transfer to the porphyrin ring which neutralizes the π-cation radical. The newly formed electron pair is of course delocalized over the porphyrin ring. Hydrogen bond formation with the ferryl oxygen atom would stabilize the newly formed H-B acid (Figure 2.6). The pK_a of this distal acid group in compound II is 8.6 ± 0.1 (21,30-32). When it is ionized compound II is unreactive.

The subsequent reaction of enol with compound II is more complicated (Figure 2.7). As in the compound I reaction, proton and electron transfer occur from the enol. However the ferryl oxygen double bond is broken, an additional proton transfer occurs and a hydrogen bond is also transferred. The net result is the reduction of iron(IV) to iron(III), and formation and departure of water from the sixth coordination position of the ferric ion. Three resonance Raman spectral studies of HRP-II have recently been completed (30-32). The pK_a of ~8.6 for the distal group is confirmed. The ferryl oxygen atom is shown to exchange with solvent water (30).

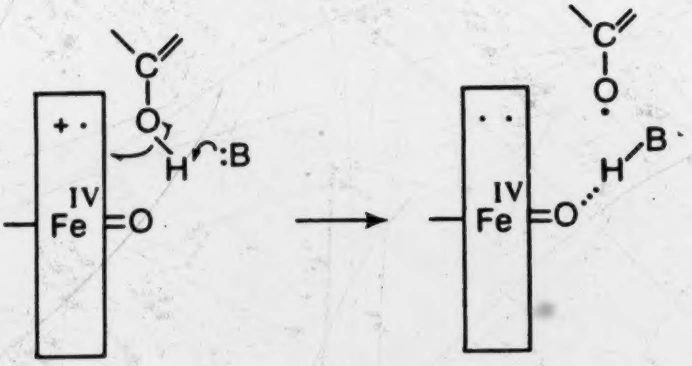

Figure 2.6. Mechanism of reaction of enol (or phenol) with HRP-I. The rectangle represents the porphyrin ring. An electron is transferred to the porphyrin ring neutralizing the π-cation radical. The newly formed electron pair is distributed around the porphyrin ring. The enol proton is transferred to a distal basic group B and the newly formed acid is stabilized by a hydrogen bond to the ferryl oxygen atom. The final enzyme structure is that of compound II.

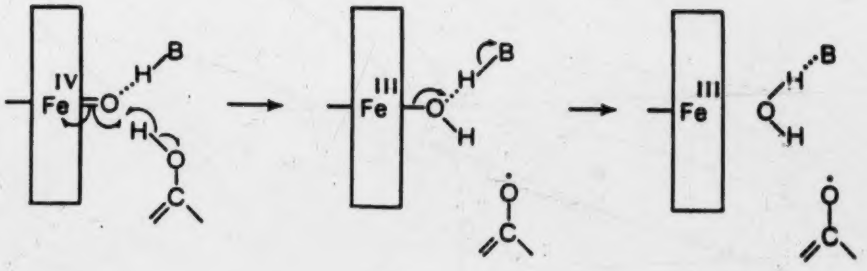
IV
Fe
O
H
B
H
O
C
III
Fe
O
H
B
H
O
C
III
Fe
H
B
O
H
O
C

Figure 2.7. Mechanism of reaction of enol (or phenol) with HRP-II. An electron is transferred to iron(IV) and two protons are transferred to the ferryl oxygen atom forming a water molecule as leaving group. The final structure is that of native HRP.

Nature has devised enzyme intermediates which accept the dominant form of the substrate in its unionized state (in the case of phenols), even though it is a poorer electron donor than its anion. In the conversion of compound I to compound II the enzyme distal base accepts the phenol (or enol) proton. This proton facilitates the exchange of the ferryl oxygen atom with bulk water, apparently by a mechanism involving OH^- departure (30). In the conversion of compound II to native enzyme another proton is accepted from a second phenol molecule which converts the ferryl OH^- into the much better H_2O leaving group.

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CHAPTER THREE

MEASUREMENT OF RATES AND EQUILIBRIA FOR KETO-ENOL TAUTOMERISM OF ALDEHYDES USING HORSERADISH PEROXIDASE COMPOUND I*

3.1 INTRODUCTION

Measurement of the relevant kinetic and thermodynamic parameters for keto-enol tautomerism of aldehydes has presented a major challenge, caused mainly by difficulties in measuring the small concentration of the enol form present in most systems (1-5). From investigations of the HRP** catalyzed reaction of isobutyraldehyde and molecular oxygen to form triplet state acetone and formic acid, it has been established that only the enol form of the aldehyde is reactive with compounds I and II of peroxidase (6-8). We report here upon exploitation of this reactivity of enols to measure the rate and equilibrium constants for the keto-enol tautomerism of IBAL, propanal, and butanal.

3.2 RESULTS AND DISCUSSION

HRP-I was prepared in close to pure form (with only inert native enzyme present as a small contaminant) and introduced into one reservoir of a stopped-flow apparatus (Photal Model RA-601) (9,10). IBAL, solubilized in up to 0.59 M aqueous ethanol, was placed into the other reservoir. All reactions were studied at 35.0°C, pH 7.4, and ionic strength 0.67 M. K_2SO_4 was used as an inert electrolyte where necessary. Phosphate buffer was used; it catalyzes the keto-enol conversion. The relevant equations are:

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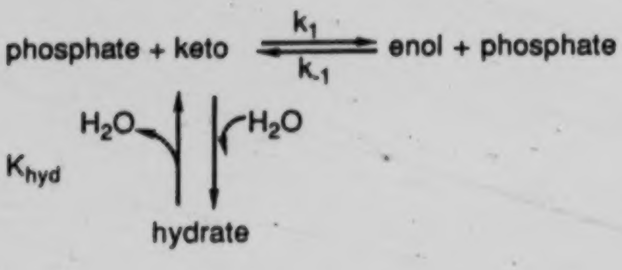
* A version of this chapter has been published. Bohne, C., MacDonald, I.D., and Dunford, H.B. (1986) *J. Am. Chem. Soc.* **108**, 7867-7868.

** Abbreviations: HRP, native horseradish peroxidase; HRP-I, compound I of horseradish peroxidase; IBAL, isobutyraldehyde; keto, keto form of isobutyraldehyde; enol, enol form of isobutyraldehyde.

[3.1]



[3.2]
$$K_{enol} = \frac{[enol]}{[keto]} = \frac{k_1}{k_{-1}}$$

[3.3]
$$\begin{aligned}[aldehyde]_{tot} &= [keto] + [hydrate] + [enol] \\ &\cong [keto] + [hydrate]\end{aligned}$$

[3.4]
$$K_{hyd} = \frac{[hydrate]}{[keto]} = \frac{[aldehyde]_{tot} - [keto]}{[keto]}$$

[3.5]
$$\therefore [keto] = \frac{[aldehyde]_{tot}}{1 + K_{hyd}}$$

Molecular oxygen does not play any role in the elementary reactions described here, only in the overall reaction (6-8).

With enol in excess with respect to compound I a pseudo-first-order reaction is observed at 411 nm, the isosbestic point between native enzyme and compound II:

[3.6]
$$\begin{aligned}-d[HRP\text{-}I]/dt &= k_{obs}[HRP\text{-}I] \\ &= k_{app}[enol][HRP\text{-}I]\end{aligned}$$

where the units of k_{obs} are s^{-1} and of k_{app} are $M^{-1}\ s^{-1}$. From equations [3.2], [3.5] and [3.6]

$$[3.7] \qquad k_{obs} = k_{app}[\text{enol}] = \frac{k_{app}K_{enol}[\text{aldehyde}]_{tot}}{1 + K_{hyd}}$$

A plot of k_{obs} *versus* total aldehyde concentration is linear with the slope equal to $k_{app}K_{enol}/(1 + K_{hyd})$. Thus if the equilibrium constants are known, the rate constant for the reaction of compound I with enol, k_{app}, can be calculated. This approach was valid for IBAL and propanal, but not for butanal because of its low solubility. The values of k_{app} are of the order of $5 \times 10^6\ M^{-1}\ s^{-1}$.

More complicated but more revealing behavior is observed when compound I is in excess of enol. Now the consumption of compound I consists of an initial burst in which the equilibrium pool of enol is consumed rapidly, followed by a slow zero-order reaction in which the rate-limiting step is the keto-enol conversion. The steady state approximation is valid in the zero-order region and it can be shown that

$$[3.8] \qquad -d[\text{HRP-I}]/dt = \frac{k_1[\text{aldehyde}]_{tot}[\text{phosphate}]}{1 + K_{hyd}} = k_{z.o.}$$

where k_1 is the second order rate constant for the conversion of keto to enol, catalyzed by phosphate and $k_{z.o.}$ is the experimentally determined zero-order rate constant (Ms^{-1}) (see Appendix B). For fixed $[\text{aldehyde}]_{tot}$ a plot of $k_{z.o.}$ *versus* [phosphate] is linear; similarly for fixed phosphate a plot of $k_{z.o.}$ *versus* $[\text{aldehyde}]_{tot}$ is linear. Therefore k_1 is calculated readily if K_{hyd} is known. Unfortunately, some measurements of K_{hyd} in the literature are inconsistent or obtained under different experimental conditions (11,12). The multiplication factor $(1 + K_{hyd})$ therefore has not been applied to the values reported in Table 3.1. We define new parameters K'_{enol} and k'_1 which are the ones listed in Table 3.1.

$$K'_{enol} = K_{enol}/(1 + K_{hyd});\ k'_1 = k_1/(1 + K_{hyd}) \tag{3.9}$$

The factor $(1 + K_{hyd})$ cancels in the determination of k^{-1}. By calibration of the stopped-flow apparatus the amount of compound I disappearing in the initial burst can be determined; this provides a measure of the equilibrium concentration of enol and hence of the equilibrium constant K'_{enol}. Since both k'_1 and K'_{enol} have been determined, k_{-1} can be calculated. A similar approach was used for propanal and butanal for which no experimentally determined constants appear in the literature. Results are summarized in Table 3.1. For IBAL, K_{enol} is 1.7×10^{-4} and k_1 $8.6 \times 10^{-5}\ M^{-1}\ s^{-1}$ with a correction for hydrate formation (13,14). These results compare favorably with those obtained using other experimental and theoretical methods (1-5).

Both propanal and butanal have cis-trans isomers in their enol forms. Our results can be fit with a single exponential curve for the burst phase which is followed by the linear zero-order phase. The burst results indicate either that there is no detectable difference in reactivity of the two geometric isomers with compound I, which would appear likely because of the known lack of selectivity in compound I reactions, or that one isomer is dominant. The observed linear behavior following the burst could be the sum of two zero-order reactions, one for each isomer.

Thus we have described a unique technique using peroxidase compound I for measuring rates and equilibria of keto-enol tautomerism which could readily be applied to a study of the influence of acid-base catalysts upon the rates.

Table 3.1. Data on Phosphate-Catalyzed Keto-Enol Tautomerism of Aldehydes and Compound I Reactions with Enols at 35°C, Ionic Strength 0.67 and pH 7.4. To Correct for Hydrate Formation, Multiply K'_{enol} and k'_1 by the factor $(1 + K_{hyd})$.

	Equilibrium Constant K'_{enol}	Rate Constants (all $M^{-1}\ s^{-1}$) keto + phosphate	enol + phosphate k_{-1}
IBAL	1.2×10^{-4}	6.0×10^{-5}	0.5
propanal	8.0×10^{-6}	1.5×10^{-4}	19
butanal	5.5×10^{-4}	1.0×10^{-4}	19

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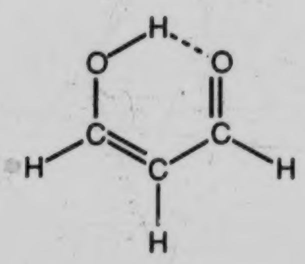
CHAPTER FOUR

MECHANISM OF HORSERADISH PEROXIDASE CATALYZED OXIDATION OF MALONALDEHYDE[*]

4.1. INTRODUCTION

Malonaldehyde is a molecule formed during lipid peroxidation (1). It has one functional group completely enolized in aqueous solution (2) so that it has the following structure:



When it is combined with HRP[**] in the presence of manganese(II) and dissolved molecular oxygen in acetate buffer, a reaction takes place in which oxygen is consumed. Previous work on this system has been centered on singlet oxygen generation (3-5). Another interesting aspect, however, is the mechanism of the peroxidase involvement in what appears to be an oxygenase reaction. Peroxidase reactions that consume dioxygen have been observed before. The HRP catalyzed oxidations of nicotinamide adenine dinucleotide (6-9), isobutyraldehyde (10-13), indoleacetic acid (14-21), linear aldehydes (22,23), phenylacetic acid (24-26) and dihydroxyfumarate (27,28) are all reactions in which dissolved molecular oxygen is consumed. In several of these cases, however, it was found that the enzyme utilizes a peroxide or peracid as the oxidizing substrate and goes through the normal peroxidative cycle. Carbon centered free radicals are generated and oxygen consumption occurs as a result of subsequent reaction of the free radical products with oxygen.

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[*] A version of this chapter has been submitted for publication in *Archives of Biochemistry and Biophysics.*

[**] Abbreviations: HRP, horseradish peroxidase (EC 1.11.1.7, donor:H_2O_2 oxidoreductase); HRP-I, HRP-II, and HRP-III are compounds I, II, and III of HRP (HRP-III is also known as oxyperoxidase); HMA, malonaldehyde showing a reactive hydrogen atom; ROOH, peracid or organic hydroperoxide; AH_2, reducing substrate; MA• and •AH, free radical oxidation products; k_{app}, apparent second-order rate constant; k_{pseudo}, pseudo-first-order rate constant.

[4.1] $$\text{HRP} + \text{ROOH} \rightarrow \text{HRP-I} + \text{ROH}$$

[4.2] $$\text{HRP-I} + \text{AH}_2 \rightarrow \text{HRP-II} + \bullet\text{AH}$$

[4.3] $$\text{HRP-II} + \text{AH}_2 \rightarrow \text{HRP} + \bullet\text{AH} + \text{H}_2\text{O}$$

[4.4] $$\bullet\text{AH} + \text{O}_2 \rightarrow \rightarrow \text{final products}$$

Note that Equation [4.1] implies oxygen atom addition occurs in compound I formation; and Equation [4.2], hydrogen atom addition in compound II formation. These reactions are well documented (29,30).

In this work we report on oxygen consumption and spectrophotometric experiments as well as steady state and transient state kinetic results on the mechanism of reaction of malonaldehyde with HRP.

4.2. MATERIALS AND METHODS

HRP, grade I, was purchased from Boehringer Mannheim as an ammonium sulfate precipitate. The stock solution was prepared by dialyzing the precipitate extensively with deionized water. The HRP concentration was determined by measuring the absorbance at 403 nm using a molar absorptivity of 1.02×10^5 $M^{-1}cm^{-1}$ (31). Malonaldehyde was prepared by a published procedure (32). Manganese(II) chloride (Gold Label) was purchased from Aldrich. Hydrogen peroxide was obtained as a 30% solution from Fisher. H_2O_2 concentrations of diluted stock solutions were determined by the peroxidase assay (33). HRP-I was prepared immediately before each experiment by combining equimolar amounts of H_2O_2 and HRP. The stability of the enzyme compound was monitored carefully by observing the Soret absorbance spectrum. All other chemicals were reagent grade and were used without further purification. For experiments on the overall reaction, acetate buffer was used with an ionic strength of 0.01 M with K_2SO_4 as an inert salt to make the total ionic strength 0.11 M. The temperature was 25°C.

The stopped-flow apparatus is a Photal Rapid Reaction Analyzer model R-601. This machine is also equipped for rapid scans of absorbance spectra through the use of a photodiode array. Rate constants for first-order reactions observed with the stopped-flow were determined by a non-linear least squares analysis. Routine absorbance spectra were determined on a Cary 219 spectrophotometer. Oxygen consumption was measured with a Yellow Springs Instrument model 53 Oxygen Monitor equipped with a Clark-type polarographic electrode. All pH measurements were made with a Fisher Accumet Model 420 digital pH meter which was calibrated with standard buffers from Fisher.

For the anaerobic experiments, enzyme solutions were deoxygenated by passing argon gas over the surface of the solution for two hours, followed by a ten minute application of ~20 torr vacuum generated from a water aspirator, followed by return to atmospheric pressure using argon. The solution container was kept in ice throughout. Argon was bubbled through other solutions for about 20 minutes followed by the vacuum procedure described above. Solutions were transferred to the stopped-flow apparatus through the use of gas-tight syringes. Screw-on reservoir caps of the stopped-flow apparatus were replaced with ones which had two-way valves. The argon line to the apparatus, which provides the driving pressure for the stopped-flow experiments, was used to flush the flow cell. Through the use of the modified reservoir caps it was possible to introduce anaerobic solutions into the reservoir chambers under positive argon pressure.

For the transient state experiments the concentration of HRP-I was 2.0 μM. Malonaldehyde concentrations were typically between 1-8 mM to give conveniently measurable kinetics. For each malonaldehyde concentration, four rate constants were measured and the median value was reported. The pH, maintained by citrate buffer was checked after the experiments. The ionic strength was 0.1 M.

4.3. RESULTS

4.3.1. Overall Reaction Using Manganese(II)

Malonaldehyde is oxidized by HRP in the presence of manganese(II) and dissolved molecular oxygen with oxygen being consumed in the reaction (3,4). Acetate buffer is required for the reaction to occur. Typical reaction conditions are acetate buffer pH 5.3, [HRP] 4.5 μM, [HMA] 3 mM, and [Mn^{II}] 3 mM. Under these conditions the oxygen is consumed in less than ten minutes. There is a lag time for reaction initiation which lasts between 5 and 25 s. The rate of oxygen consumption was maximized when the concentration of manganese(II) was roughly twice the malonaldehyde concentration (Table 4.1). The concentration of acetate required to maximize the rate of oxygen consumption was roughly 0.03 M (Figure 4.1). In all cases the oxygen consumption proceeded to completion.

4.3.2. Overall Reaction Using Hydrogen Peroxide

We found that it was possible to induce oxygen consumption by replacing manganese(II) by hydrogen peroxide. Hydrogen peroxide also reduced the lag time. The degree of oxygen consumption is linearly dependent on the amount of H_2O_2 added and the stoichiometry of moles oxygen consumed per mole hydrogen peroxide added is 2:1 (Figure 4.2). The acetate buffer can be replaced with citrate buffer for the H_2O_2 reaction.

4.3.3. Horseradish Peroxidase Compound I Reaction

When HRP-I was reacted with malonaldehyde at pH 4.0, citrate buffer, ionic strength 0.1 M, [HMA] 0.5 mM, the amount of oxygen consumed was linearly dependent on the amount of HRP-I present (Figure 4.3). The stoichiometry of O_2 consumed to HRP-I reacted is 2:1.

The spectral changes observed during the HRP-I reaction with malonaldehyde indicate that HRP-I is being converted to oxyperoxidase, HRP-III (Figure 4.4). The peak seen in the Soret region at 413 nm using the rapid-scan apparatus and the two absorbance maxima in the visible region at 543 nm and 577 nm observed on the Cary spectrophotometer are very close to the literature values for HRP-III: 416 nm, 546 nm, and 583 nm (34). The small differences in wavelength are likely due to the presence of a small amount of native HRP

Table 4.1. Rate of Oxygen Consumption as a Function of Manganese(II) Concentration[a]

$[Mn^{II}]$, mM	Rate of O_2 Consumption, µM O_2/s
0	0
0.005	0
0.05	0
0.49	0
0.98	0.67
1.97	2.35
2.95	3.28
3.93	4.39
4.92	4.21
5.90	4.68
6.89	5.15
7.87	4.78
8.85	4.86
9.84	4.86

(a) Acetate buffer, pH 5.3, [HRP] 3.6 µM, [HMA] = 2.9 mM, t 25°C.

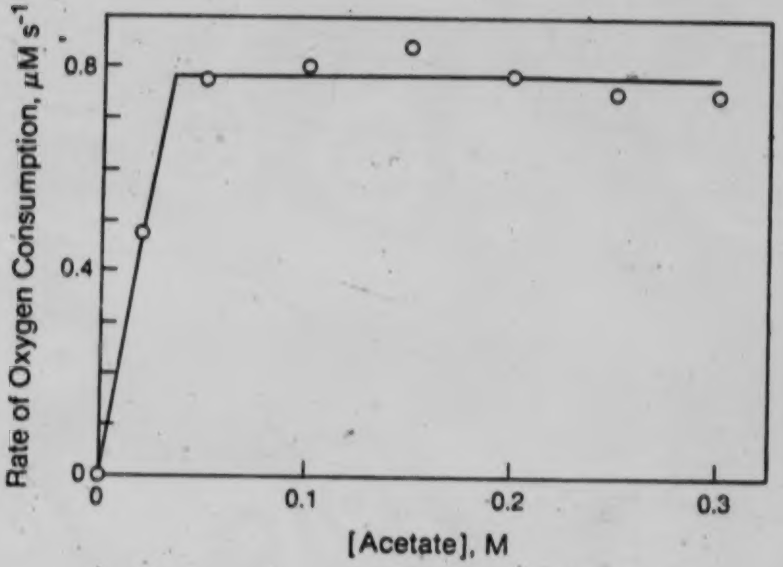


Figure 4.1. Plot of rate of oxygen consumption *versus* [acetate] for the HRP/HMA/Mn^{II}/O$_2^-$ reaction system, ionic strength 0.11 M, pH 4.5, [HMA] 0.93 mM, [Mn^{II}] 1 mM, [HRP] 1 µM, temperature 25°C.

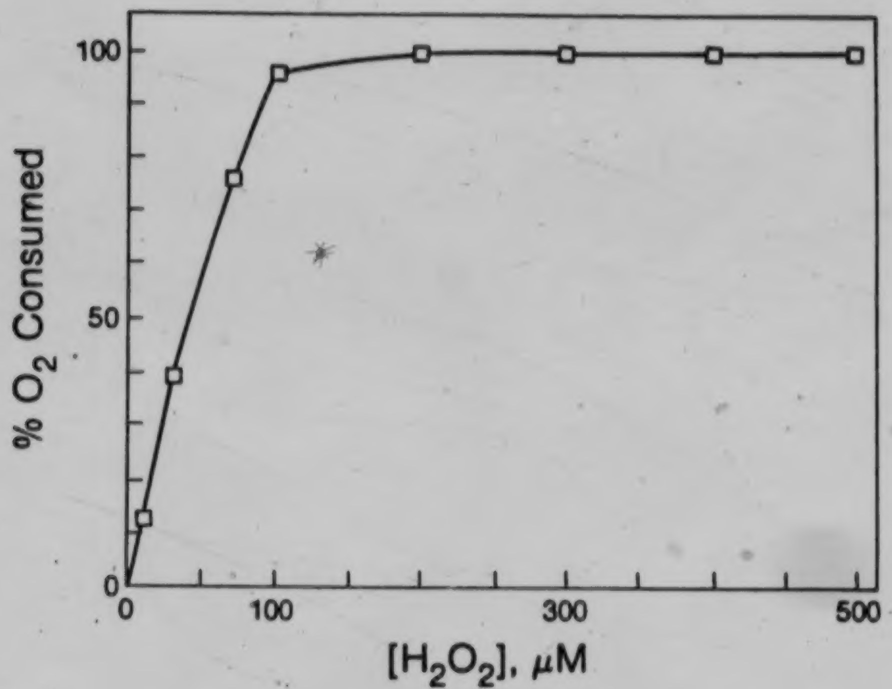


Figure 4.2. Plot of percent oxygen consumed *versus* [H_2O_2] for the HRP/HMA/H_2O_2/O_2 reaction system in acetate buffer, ionic strength 0.11 M, pH 5.3, [HRP] 4.4 μM, [HMA] 2 mM, temperature 25°C.

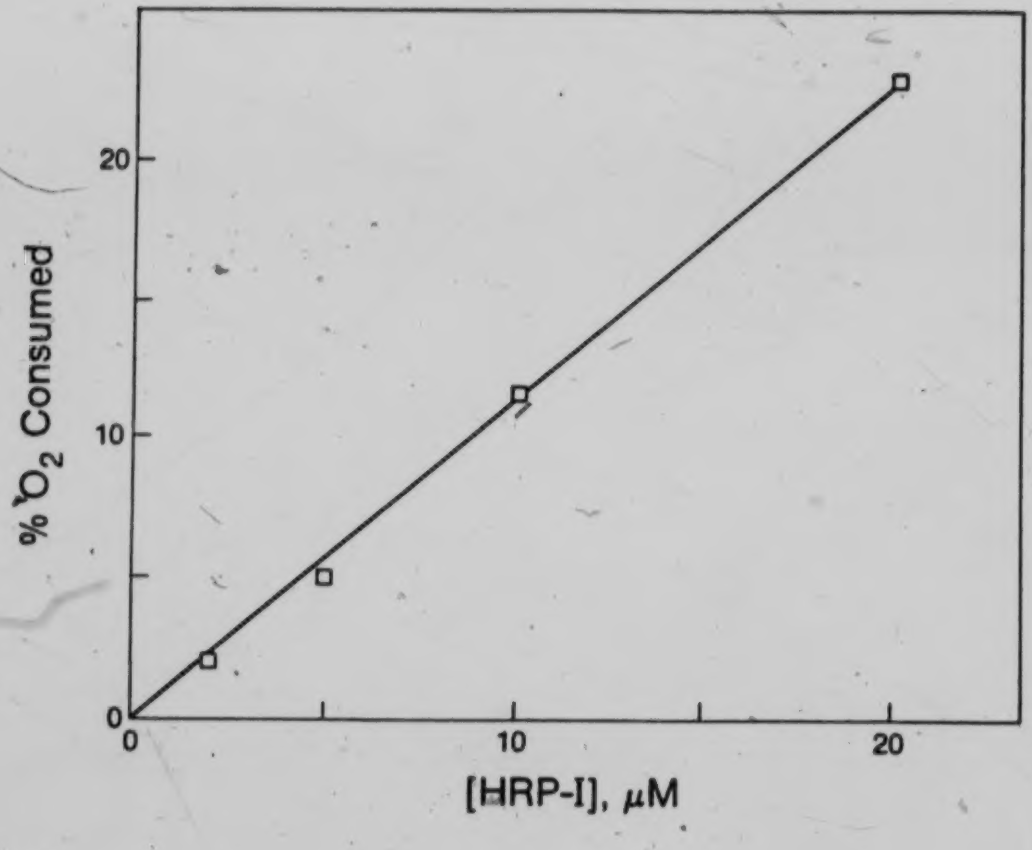


Figure 4.3. Plot of percent oxygen consumed *versus* [HRP-I] for the reaction of HRP-I with HMA in the presence of dissolved molecular oxygen. Reactions were carried out in citrate buffer, ionic strength 0.10 M, pH 4.0, [HMA] 0.32 mM, temperature 25°C.

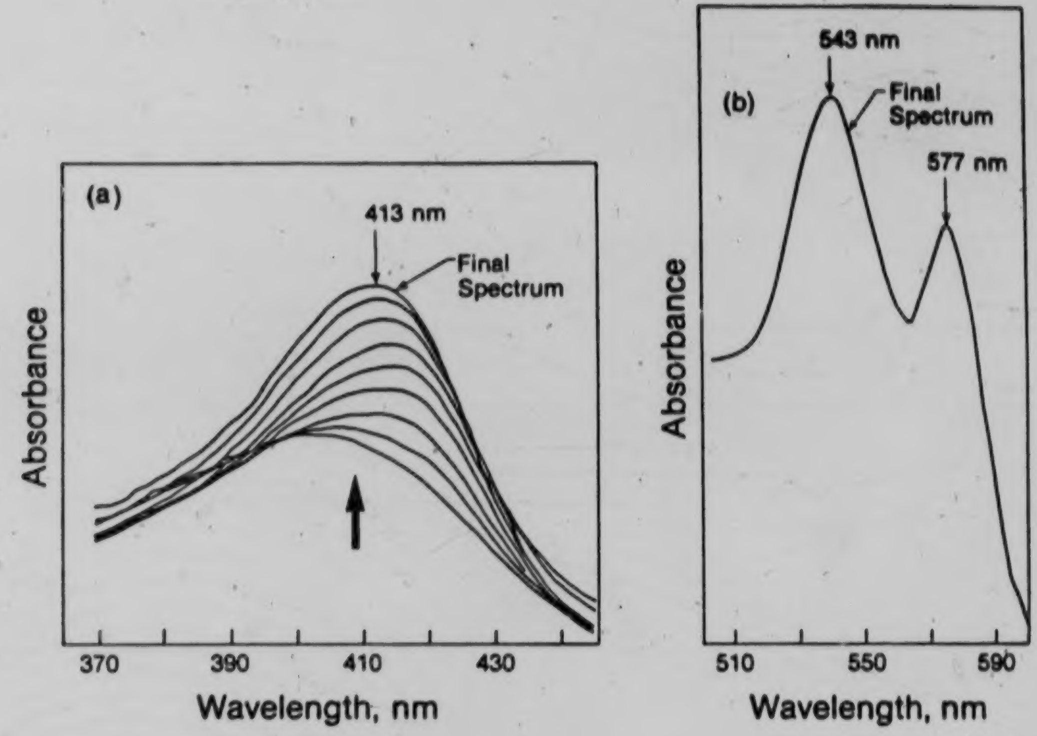


Figure 4.4. (a) Rapid scan Soret spectra of the reaction of HRP-I with HMA under aerobic conditions. The reaction was carried out in citrate buffer, ionic strength 0.10 M, pH 4.0, [HMA] 2 mM, [HRP-I] 4.0 μM, temperature 25°C. Starting with the lowest spectrum, the timing of the spectra are 0.15, 0.35, 0.65, 1.25, 1.85, 2.75, 4.55, 8.30, and 50 s after mixing.

(b) Visible spectrum of the final reaction mixture taken under identical experimental conditions as (a) but using the Cary spectrophotometer.

which would shift the peaks to shorter wavelength. HRP-III formation was prevented by operating under anaerobic conditions as shown by the rapid-scan experimental results (Figure 4.5). Instead of HRP-III formation, HRP-I is converted to HRP-II followed by reaction of HRP-II back to native enzyme. The isosbestic point observed at 411 nm is characteristic of an HRP-II-to-native-HRP reaction.

In order to study the kinetics of the elementary reaction of HRP-I with malonaldehyde it was necessary to operate under anaerobic conditions to avoid spectral interference that would arise from the accumulation of HRP-III. We monitored the rate of disappearance of HRP-I by the increase in absorbance at 411 nm, the isosbestic point of HRP/HRP-II. With malonaldehyde in excess of HRP-I, the reaction proceeds by pseudo-first-order kinetics (Figure 4.6). The second-order rate constant was obtained for the linear slope of the plot of the first-order rate constant *versus* [malonaldehyde] (Figure 4.7). These experiments were repeated at several pH's over the range of 3.5-4.8 (Table 4.2). The mean value of the eight rate constants in Table 4.2 is 88 ± 6 M^{-1} s^{-1}. At pH values above 5 the rate decreases and an accurate determination of the rate constants were not possible.

4.4. DISCUSSION

Oxygen consumption still occurs when manganese(II) is replaced by hydrogen peroxide (Figure 4.2). This suggests that a peroxide is being generated in the overall reaction. The presence of peroxide with HRP strongly implies the involvement of HRP-I. The rate constant for reaction of peroxides with HRP is typically ~10^7 M^{-1} s^{-1} (29,30).

The involvement of HRP-I and HRP-II is supported by the oxygen consumption results. When hydrogen peroxide is added to the reaction system

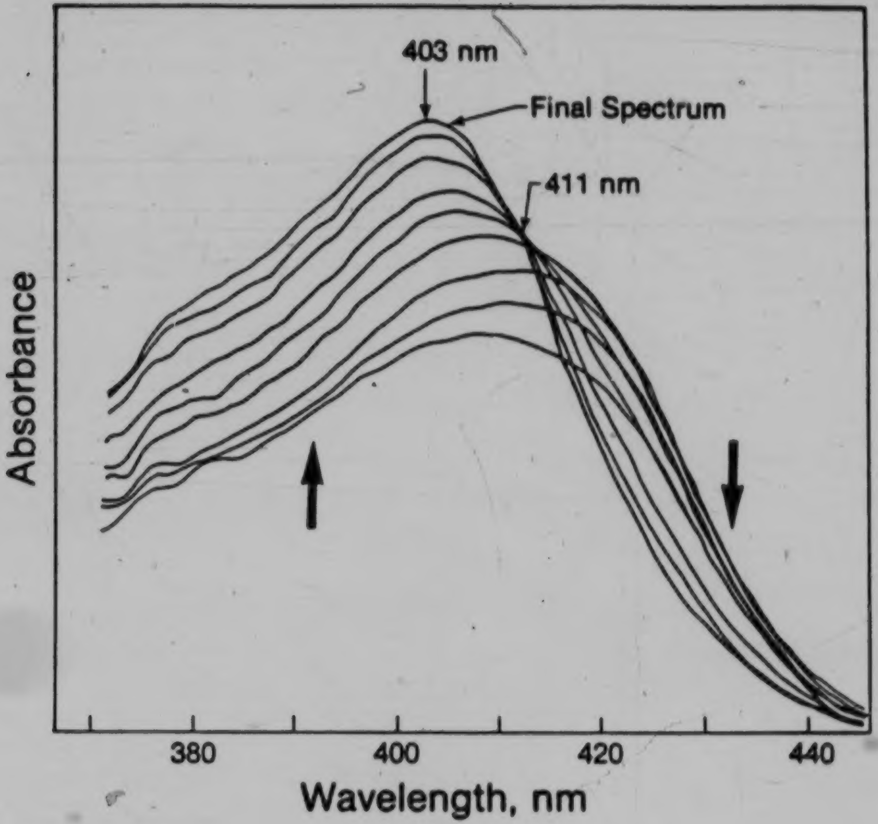


Figure 4.5. Rapid scan Soret spectra of the reaction of HRP-I with malonaldehyde under anaerobic conditions. The reaction was carried out in citrate buffer, ionic strength 0.1 M, pH 4.0, [HMA] 1.34 μM, [HRP-I] 2.0 μM, temperature 25°C. Starting with the lowest spectrum, the timing of the spectra are 0.15, 0.30, 0.80, 1.15, 2.25, 3.35, 5.55, 8.30, and 120 s after mixing.

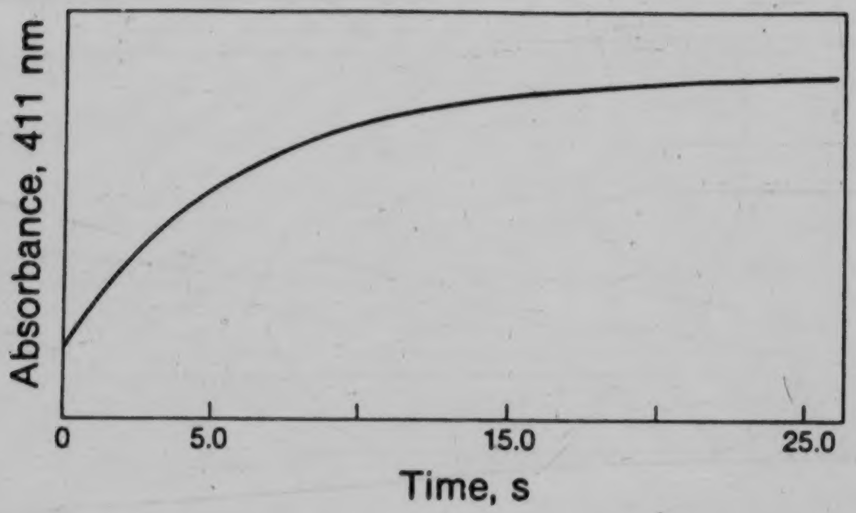


Figure 4.6. Stopped-flow trace of the absorbance at 411 nm for the reaction of HRP-I with HMA under anaerobic conditions in citrate buffer, ionic strength 0.10 M, pH 3.91, [HRP-I] 20 μM, temperature 25°C.

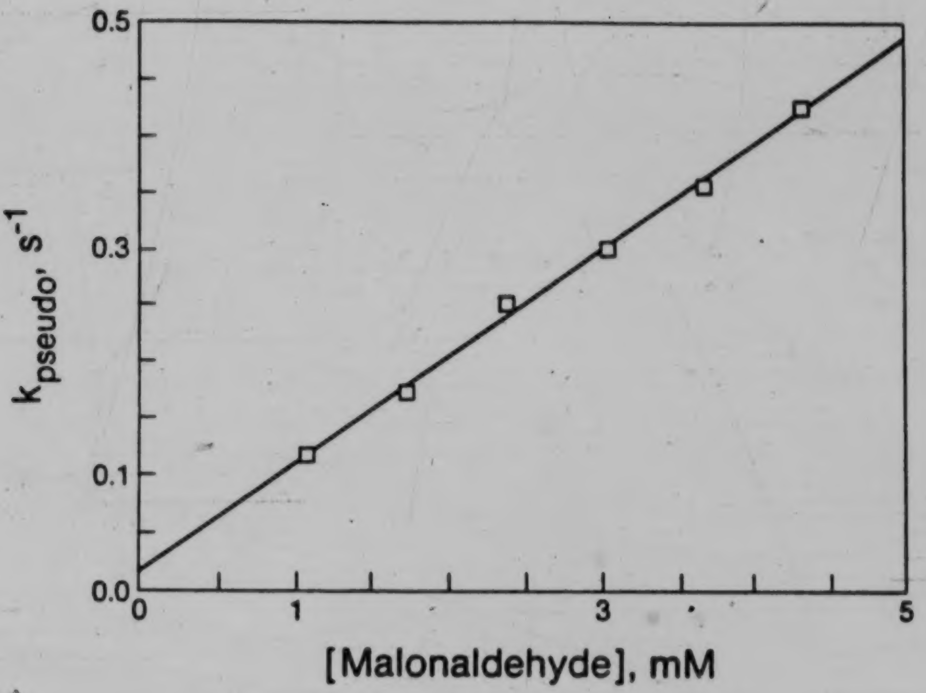


Figure 4.7. Plot of k_{pseudo} *versus* [Malonaldehyde] under the same reaction conditions as described for Figure 4.6.

Table 4.2. Second-Order Rate Constant as a Function of pH

pH	k_{app} ± s.d. *, M^{-1} s^{-1}
2.44	104 ± 3
3.67	84 ± 4
3.79	85 ± 4
3.92	94 ± 3
4.12	83 ± 10
4.35	83 ± 5
4.68	88 ± 9
4.80	80 ± 6

* s.d. = standard deviation from linear least squares analysis.

in place of manganese(II), the extent of reaction is limited by the concentration of peroxide added; the stoichiometry is two moles oxygen consumed per mole hydrogen peroxide added (Figure 4.2). This stoichiometry is expected if hydrogen peroxide reacts with native enzyme to give HRP-I for the following reasons. HRP-I can react with HMA yielding HRP-II and a free radical of HMA. HRP-II can also react with HMA giving the same radical and HRP in its native state. Therefore a single turnover of the enzyme generates two radicals each of which scavenge a molecule of oxygen accounting for the 2:1 stoichiometry of the overall reaction.

When HRP-I is prepared directly and reacted with HMA with no added peroxide or manganese(II), oxygen consumption occurs but again is limited, this time by the concentration of HRP-I. This shows that it is HRP-I formation which is responsible for oxygen consumption. The stoichiometry of two moles of oxygen consumed per mole of HRP-I reacted demonstrates that HRP-II must also be involved producing HMA radicals which go on to react with dissolved molecular oxygen (Figure 4.3).

The rapid-scan results for the HRP-I-malonaldehyde reaction in the Soret region show that, under anaerobic conditions HRP-I is being converted to HRP-II which in turn reacts to form native enzyme (Figure 4.5). These facts point to the role of HRP as a normal peroxidase; the oxygen consumption results from scavenging of peroxidase-generated free radicals.

[4.1] $$\text{HRP} + \text{ROOH} \rightarrow \text{HRP-I} + \text{ROH}$$

[4.5] $$\text{HRP-I} + \text{HMA} \rightarrow \text{HRP-II} + \text{MA}\bullet$$

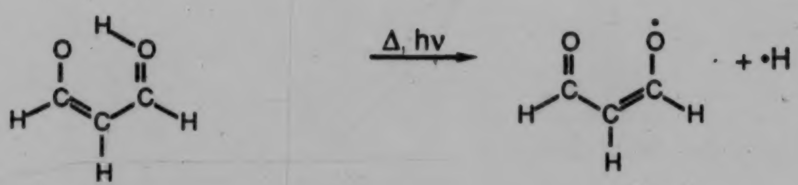
[4.6] $$\text{HRP-II} + \text{HMA} \rightarrow \text{HRP} + \text{MA}\bullet$$

[4.7] $O_2 + MA\bullet \rightarrow \rightarrow \text{final products}$

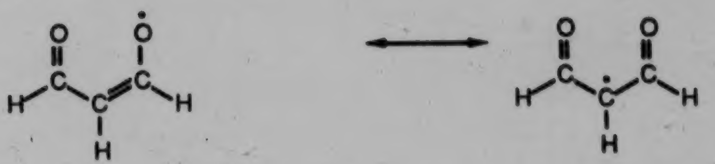
Oxygen consumption from an oxygenated solution is observed in the following reaction mixtures: (i) HRP + malonaldehyde + manganese(II) + acetate buffer; (ii) HRP + malonaldehyde + hydrogen peroxide, and (iii) HRP-I + malonaldehyde. However the behavior is very different in case (i) compared to (ii) and (iii). For the reaction mixture containing manganese(II) there is both a concentration- and time-dependent induction. Until sufficient manganese(II) is added there is no reaction (Table 4.1). As soon as a critical [Mn^{II}] is reached the oxygen consumption reaction goes to completion, but the optimum rate of oxygen consumption increases linearly with [Mn^{II}] until a second critical [Mn^{II}] is surpassed (Table 4.1). The temporal induction period becomes shorter with increased [Mn^{II}]. In cases (ii) and (iii), the extent of oxygen consumption is directly proportional to [H_2O_2] and [HRP-I], respectively, and there is no induction period. These results imply that manganese(II) is responsible for production of an HRP oxidant which propagates the peroxidase cycle. A chain reaction does not take place in the absence of manganese(II). A catalytic role for the transition metal ion is not likely since manganese(II) must be roughly equimolar with malonaldehyde for a maximal rate.

One possible mechanism for manganese(II) assisted generation of an oxidant for the peroxidative cycle is a manganese assisted autoxidation process.

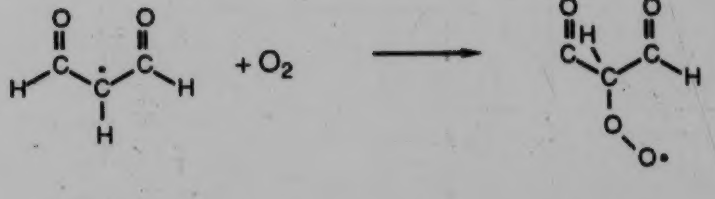
[4.8]



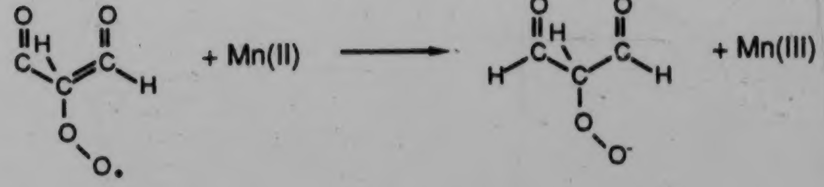
[4.9]



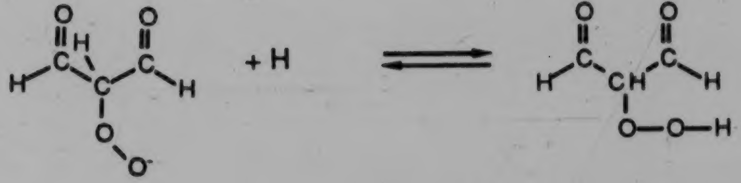
[4.10]



[4.11]



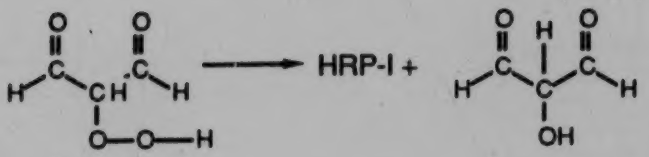
[4.12]



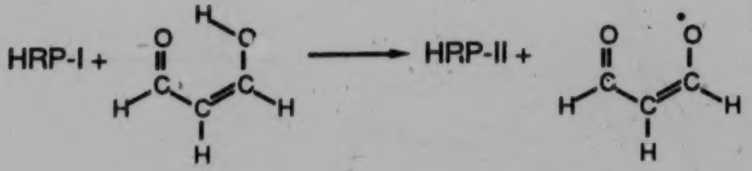
The reduction of peroxyl radicals by manganese(II) occurs with a rate constant of ~2×10^5 M^{-1} s^{-1} (35).

Thus a tentative overall mechanism for the apparent oxygenase reaction of HRP with malonaldehyde may be summarized as follows. The initiation reaction occurs from the slow autoxidation of malonaldehyde in the presence of manganese(II) to generate a small amount of peroxide (Equations [4.8]-[4.12]). This initiates the participation of HRP to generate free radicals:

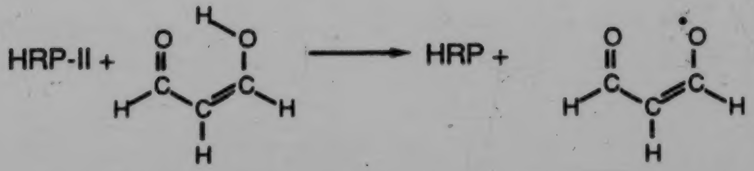
[4.13]



[4.14]



[4.15]



Reactions [4.13]-[4.15] are followed by oxygen consumption and peroxide regeneration (Equations [4.9]-[4.12]).

The role of acetate ion in this system is not clear although complex formation with manganese(II) is possible. It has been reported that at 25°C with an ionic strength of 0.1 M the acetate/manganese(II) complex formation constant is 6.31 ± 0.06 M^{-1} for the 1:1 complex (36). The complexation of manganese(II) may make it reactive enough to reduce the peroxyl radical.

That malonaldehyde exists with one functional group completely in its enol form is well established. An intramolecular hydrogen bond completes a six-membered ring structure which leads to its low reactivity (2,37,38). The reactive form of malonaldehyde is probably the enol, not the enolate anion nor the keto form, present in negligible concentration. This would agree with previous work on HRP reactions with other aldehydes and phenols (13,39). The system is very sluggish with a low reaction rate for malonaldehyde. The comparative reactivity of hydrogen peroxide is so great that it is capable of totally replacing the complicated chain process which generates organic hydroperoxide. The peroxyl radical derived from malonaldehyde also appears to be unreactive. Thus manganese(II) is an effective reductant of these radicals, replacing the pathway of hydrogen atom abstraction to generate peroxide. Recombination of peroxyl radicals to generate a tetraoxide intermediate would account for the singlet oxygen detected in this system (3,4).

Malonaldehyde is often regarded as the end product of lipid peroxidation. However results of the present study show that even this relatively inert molecule is capable of participating in free-radical-generating reactions which can lead to further oxidative damage particularly at low pH. The reactivity of malonaldehyde with amino acids, proteins, guanine, and guanine nucleosides has been demonstrated (40-43) but its mutagenicity has apparently been overestimated (44).

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CHAPTER FIVE

KINETICS AND EQUILIBRIA OF CYANIDE BINDING TO PROSTAGLANDIN H SYNTHASE[*]

5.1. INTRODUCTION

PGH synthase[**] catalyzes the initial steps in the reactions of arachidonic acid to form the prostaglandins, thromboxanes, and prostacyclin (1-3). The enzyme is responsible for the cyclooxygenation and oxygenation of arachidonic acid to prostaglandin G_2 and the peroxidase reaction which reduces the peroxide to prostaglandin H_2 (4,5). The optical spectral properties of PGH synthase are similar to those of horseradish peroxidase (6,7). In this chapter we report the results of an equilibrium and kinetic study of cyanide binding which exploits these spectral properties to characterize further this important enzyme.

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[*] A version of this chapter has been submitted for publication.

[**] Abbreviations: PGH synthase, prostaglandin H synthase (EC 1.14.99.1); DDC, diethyldithiocarbamate; Tris, tris(hydroxymethyl)methylamine; λ_{max}, wavelength of maximum absorbance; k_{obs}, pseudo-first-order rate constant; k_{+app}, apparent second-order binding rate constant; k_+, pH independent second-order binding rate constant; k_{-app}, apparent first-order dissociation rate constant; k_-, pH independent first-order dissociation rate constant; K_{app}, apparent heme-linked enzyme acid dissociation equilibrium constant; K_a, actual heme-linked enzyme acid dissociation equilibrium constant; K_d, dissociation equilibrium constant for cyanide binding to prostaglandin H synthase; E, unprotonated form of prostaglandin H synthase; EHAc, acetate-bound form of prostaglandin H synthase; EHCN, cyanide bound form of prostaglandin H synthase; K_1, dissociation equilibrium constant for acetate binding to the protonated form of prostaglandin H synthase; K_2, dissociation equilibrium constant for acetic acid binding to the unprotonated form of prostaglandin H synthase; K_{ac}, acid dissociation equilibrium constant for acetic acid; HAc, acetic acid; Ac^-, acetate anion; HRP, horseradish peroxidase.

5.2. MATERIALS AND METHODS

All reagents used for buffers were of the highest grade available. Potassium cyanide was from Fisher. Its purity was confirmed by silver nitrate titration; subsequently solution concentrations were determined by weight. Arachidonic acid was from Nu-Chek-Prep; hematin from Calbiochem; DDC from Sigma Chemical Co.; phenol from British Drug House; DE53 ion-exchange chromatography gel from Whatman; Tween 20 from J.T. Baker Chemical Company. Protein assays were carried out by the Bio-Rad protein assay method. Cyclooxygenase activity was measured by monitoring oxygen consumption for the reaction of arachidonic acid with PGH synthase in the presence of 1 µM hematin, 1 mM phenol, 200 µM arachidonic acid, 0.1 M phosphate buffer, pH 8.0 at 30°C. The reaction was initiated by the addition of a small volume of concentrated arachidonic acid. PGH synthase concentrations were typically around 0.1 µM in these assays. One unit of activity is defined as 1 µmole arachidonic acid oxidized per minute.

PGH synthase was isolated from sheep seminal vesicles which were obtained from a local slaughterhouse. The vesicles were frozen in dry ice within 20 minutes of slaughter. They retain full enzyme activity for at least one year if kept on dry ice. The isolation procedure is a modification of that of Marnett *et al.* (8). There are three modifications. The first is the use of a batch procedure for the application of the solubilized protein to the ion-exchange DE53 gel. This protein-loaded gel is then transferred from a beaker to a column and eluted according to Marnett's procedure. The second modification was the use of 5 mM rather than 10 mM Tris buffer for the solubilization of the protein and equilibration of the ion exchange gel. The last change was the use of 0.1 mM instead of 0.3 mM DDC in the elution of the enzyme from the DE53 column.

All other reagent concentrations and conditions were as described by Marnett (8).

The enzyme is isolated as a roughly 50/50 mixture of apo- and holoenzyme. Full activity is restored by the addition of excess hematin. In this study no hematin was added to the enzyme. The specific activity of the purified enzyme is roughly 20 units per milligram protein in the absence of exogenous hematin. The enzyme is >95% pure according to sodiumdodecyl sulfate polyacrylamide gel electrophoresis.

Routine spectra were recorded on a Cary 219 spectrophotometer. Rapid-scan and stopped-flow experiments were carried out on a Photal Rapid Reaction Analyzer, Model 601. Pseudo-first-order rate constants were determined by a curve fitting analysis carried out by computer. Oxygen consumption was measured with a Yellow Springs Instrument Model 53 Oxygen Monitor equipped with a Clark-type polarographic electrode. A Fisher Accumet Model 420 digital pH meter was used for pH measurements along with Fisher standard buffers.

The stability of the enzyme in various buffer systems was checked by monitoring the Soret peak at 412 nm as a function of time on the Cary spectrophotometer at 25°C.

All experiments on cyanide binding were conducted at a temperature of (4± 1)°C, ionic strength of 0.22 M with buffer accounting for 0.10 M and K_2SO_4 accounting for 0.12 M, and 100 μM DDC. The pH range of the study was 3.96-8.00. The pH of the reaction mixtures was checked after each experiment. The cyanide stock solutions were always prepared immediately prior to use. For the lower pH values cyanide solutions were titrated with small volumes of concentrated HCl to obtain the desired pH.

5.3. RESULTS

The experiments were carried out in the presence of 100 µM DDC because of the stabilizing effect it has on the enzyme as shown by its spectral properties (Figure 5.1).

5.3.1. Kinetics of Cyanide Binding

Cyanide binding to PGH synthase results in a rapid spectral shift in λ_{max} from 412 nm to 424 nm with an isosbestic point at 421 nm (Figure 5.2). After four minutes λ_{max} of the cyanide complex is shifted to 427 nm with a slightly lower absorbance value. The cyanide binding process was measured kinetically by monitoring the absorbance change at 412 nm under pseudo-first-order conditions with cyanide in excess (Figure 5.3). The pseudo-first-order rate constant, k_{obs}, was calculated by a non-linear least squares curve fitting analysis carried out by computer. The calculated curve is shown by the broken line in Figure 5.3. The k_{obs} values were measured as a function of cyanide concentration. A plot of k_{obs} *versus* [HCN] gives a straight line (Figure 5.4) which fits the following equation:

[5.1] $$k_{obs} = k_{+app}[\mathrm{HCN}]$$

Ordinarily for a ligand binding study a plot of k_{obs} *versus* [ligand] done for experiments under pseudo-first-order conditions gives a finite intercept which is equivalent to the dissociation or "off" rate constant.

[5.2] $$k_{obs} = k_{+app}[\mathrm{ligand}] + k_{-app}$$

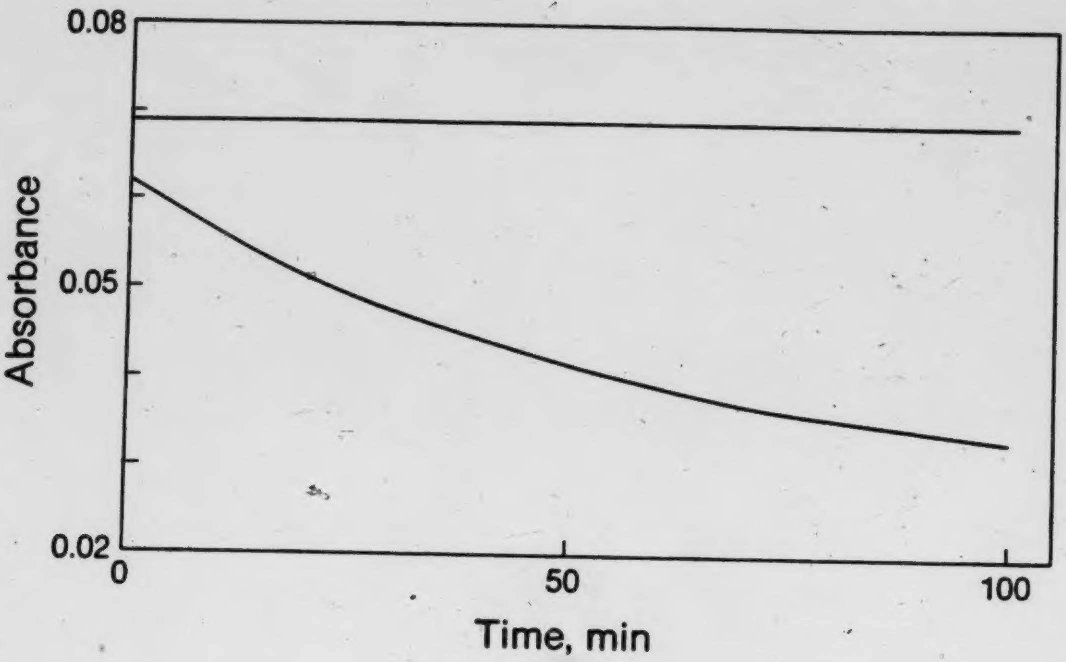


Figure 5.1. Plot of absorbance at 412 nm *versus* time for PGH synthase at pH 8.0, Tris buffer, ionic strength 0.22 M, at 4°C. Top line is the plot when the enzyme is in 100 µM DDC, 0.1% Tween 20. Bottom line is the plot when the enzyme is in 30% glycerol.

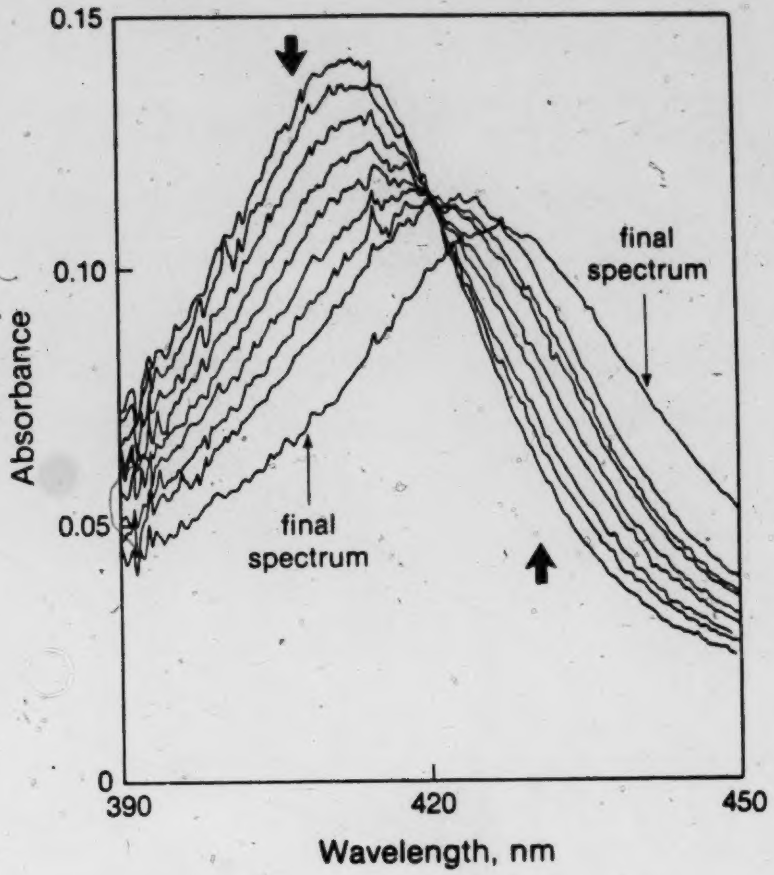


Figure 5.2. Plots of absorbance *versus* wavelength for 1.0 µM PGH synthase reacting with 8 mM HCN at 4°C in Tris buffer, pH 8.0, 100 µM DDC, 0.1% Tween 20, ionic strength 0.22 M. The thick arrows indicate the direction of the absorbance change with increasing time. Times of the spectra are 0, 1, 11, 21, 31, 51, 71, and 131 milliseconds after mixing. The final spectrum was taken 4 minutes after mixing.

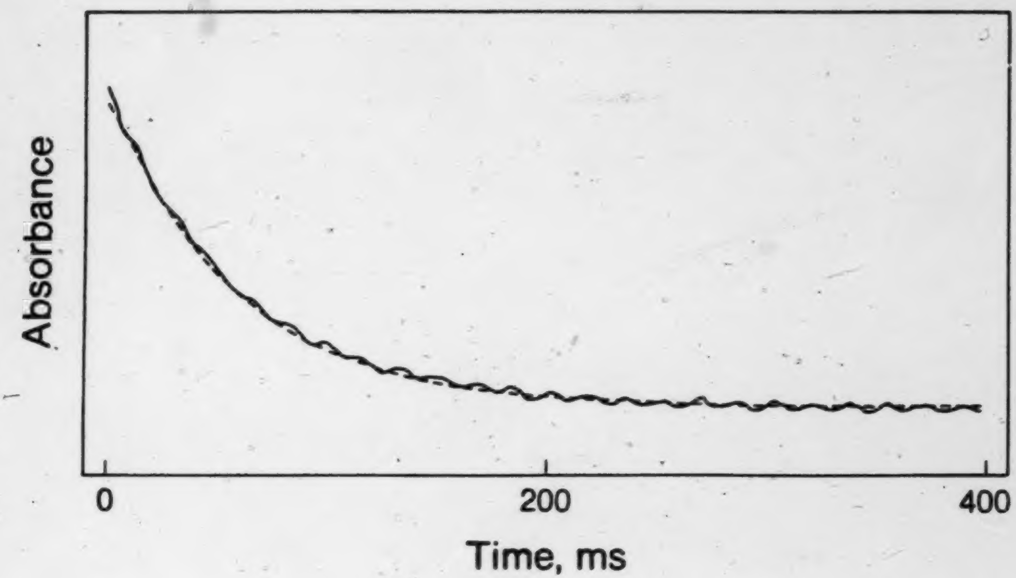


Figure 5.3. Plot of absorbance at 412 nm *versus* time for the reaction of 0.4 µM PGH synthase with 6 mM HCN. All other reagents and conditions as in Figure 5.2. Broken line is computed exponential curve fit.

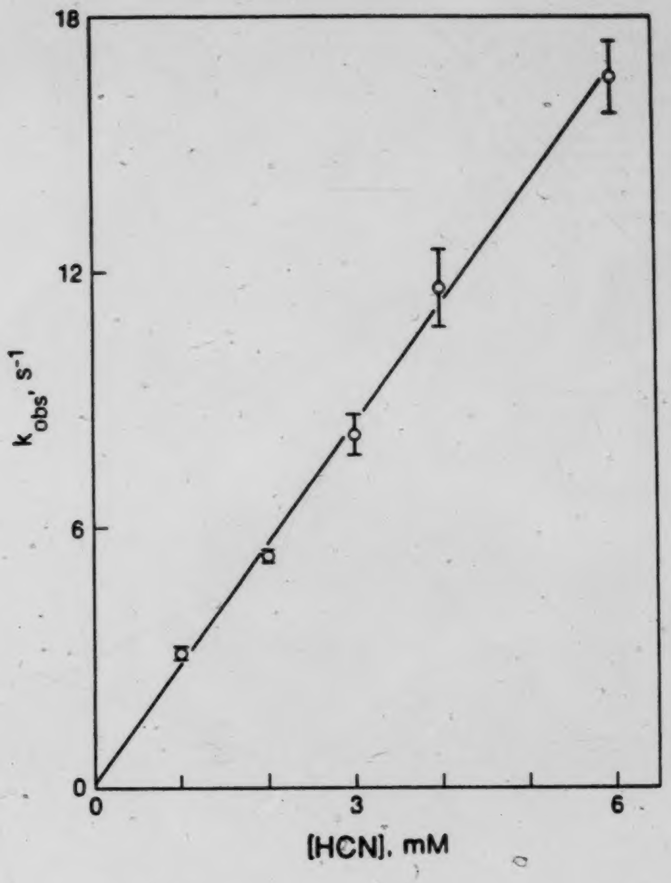


Figure 5.4. Plot of pseudo-first-order rate constant *versus* HCN concentration for the reaction of 0.4 µM PGH synthase. All other reagents and conditions as in Figure 5.2. The slope of the line is $(2.8 \pm 0.2) \times 10^3\ M^{-1}\ s^{-1}$ and the intercept is $(0.15 \pm 0.34)\ s^{-1}$.

Under our experimental conditions the value for k_{-app} is (0.15 ± 0.34) s^{-1} which is zero within experimental error. The value of k_{+app} is $(2.8 \pm 0.2) \times 10^3\ M^{-1}\ s^{-1}$. Normally the dissociation equilibrium constant K_d can be determined from the ratio of the "off" over the "on" rate constants. Because of the large error in k_{-app} such a calculation of K_d cannot be done in this case.

A series of individual experiments were performed at various pH's within the range 3.96-8.00 in which the second-order k_{+app} value was calculated by dividing the pseudo-first-order rate constant by the cyanide concentration. A plot of log k_{+app} *versus* pH is shown in Figure 5.5. In the pH range from 4 to 5, different results are obtained with two different buffers. Both sets of data could be fit to an equation based upon the assumption that an acid dissociation in the active site of the enzyme is kinetically important.

There are two possible mechanisms of cyanide binding to the enzyme. It could be hydrocyanic acid binding to a basic form of the enzyme or cyanide anion binding to an acidic form of the enzyme. The only method of eliminating one is if it leads to a k_{+app} which is larger than the diffusion controlled limit. At pH 4.12 with citrate buffer the hydrocyanic acid concentration is 8.0×10^{-3} M and the cyanide anion concentration is 3.3×10^{-8} M. These concentrations were calculated using a pK_a of 9.51 for HCN at 4°C at an ionic strength of 0.22 M. This pK_a value was determined by extrapolation of data obtained in a temperature dependence study of the pK_a of HCN to 4°C (9) followed by correction for ionic strength through the use of the extended Debye-Hückel equation. The pseudo-first-order rate constant is 13 ± 3 s^{-1}. This gives a k_{+app} of $(1.6 \pm 0.4) \times 10^3\ M^{-1}\ s^{-1}$ for the hydrocyanic acid mechanism or $(4 \pm 1) \times 10^8\ M^{-1}\ s^{-1}$ for the cyanide anion binding mechanism. The value for anion binding is close to, but not above, the diffusion controlled limit of $10^9\ M^{-1}\ s^{-1}$ for reaction of enzymes with small molecules (10). Therefore the cyanide anion binding

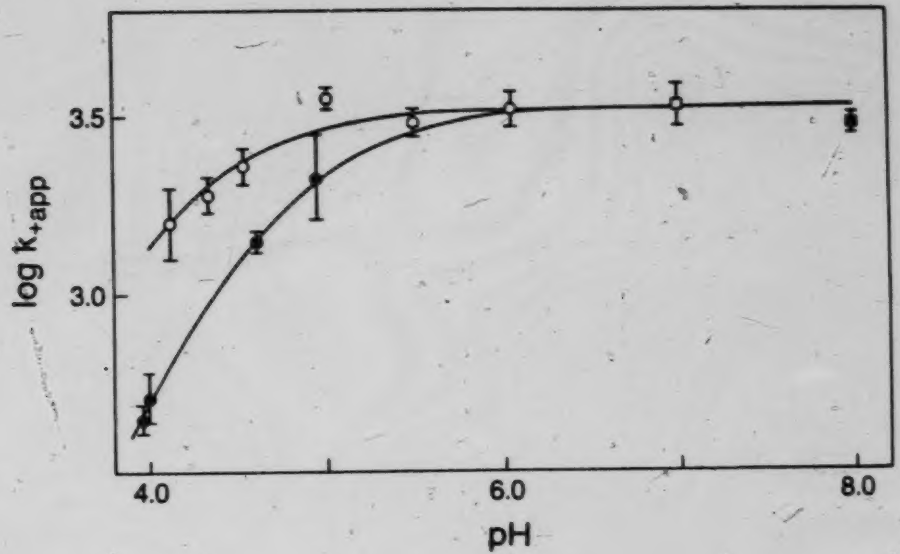
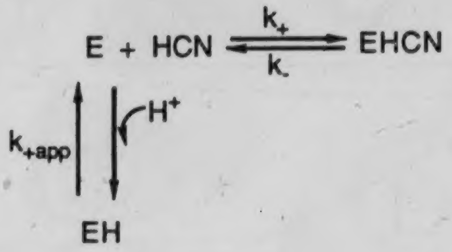


Figure 5.5. Plot of log k_{+app} *versus* pH for the reaction of 0.4 µM PGH synthase with cyanide in 100 µM DDC, 0.1% Tween 20, ionic strength 0.22 M at 4°C. -■- Tris buffer; -□- phosphate buffer; -●- acetate buffer; -O- citrate buffer. The lines on the graph are the non-linear least squares computed curve fits. For the fit with the points between pHs 4 and 5 being in acetate buffer the parameters are: $k = (3.4 \pm 0.1) \times 10^3\ M^{-1}\ s^{-1}$ and $K_{app} = (1.75 \pm 0.10) \times 10^{-5}$ M or $pK_{app} = 4.75 \pm 0.03$. For the fit with the points between pH's 4 and 5 being in citrate buffer the parameters are: $k = (3.4 \pm 0.2) \times 10^3\ M^{-1}\ s^{-1}$ and $K_{app} = (7 \pm 1) \times 10^{-5}$ M or $pK_{app} = 4.15 \pm 0.10$.

mechanism cannot be rejected outright; however in our opinion the hydrocyanic acid mechanism is favored. Studies on cyanide binding by other heme peroxidases; HRP (11), myeloperoxidase (12), and chloroperoxidase (13), have shown that it is hydrocyanic acid reacting with the basic form of the enzyme. Therefore the minimum scheme required to fit the two sets of data, obtained with acetate and citrate buffer, is:

[5.3]



The resulting equation for k_{+app} is:

[5.4]

$$k_{+app} = \frac{k_+}{1 + \frac{[H^+]}{K_{app}}}$$

For the set of data obtained with acetate buffer the best-fit values for the parameters are $k_+ = (3.4 \pm 0.1) \times 10^3\ M^{-1}\ s^{-1}$ and $K_{app} = (1.75 \pm 0.10) \times 10^{-5}$ M or $pK_{app} = 4.75 \pm 0.03$. For citrate buffer the values are $k = (3.4 \pm 0.2) \times 10^3$ $M^{-1}\ s^{-1}$ and $K_{app} = (7 \pm 1) \times 10^{-5}$ M or $pK_{app} = 4.15 \pm 0.10$. The errors are the standard deviations obtained from the curve fits.

5.3.2. Equilibrium Constant for Cyanide Binding

Cyanide binding to PGH synthase forms a 1:1 complex as indicated by the linear Scatchard plot shown in Figure 5.6. The absorbance changes resulting from addition of various cyanide concentrations to PGH synthase are plotted according to the Scatchard equation (14)

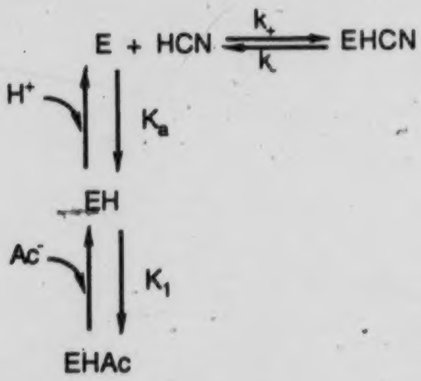
[5.5]
$$\frac{(A_0\text{-}A)}{[E]_0}\,\frac{1}{[S]}=\frac{\Delta\varepsilon}{K_d}\,\frac{(A_0\text{-}A)}{[E]_0}$$

where A_0 is the absorbance of the enzyme at a particular wavelength in the absence of ligand and A is the absorbance in the presence of ligand. The total enzyme concentration is $[E]_0$ and $\Delta\varepsilon$ is the difference in molar absorptivity between the native enzyme and complex at 412 nm. The value for the dissociation constant was determined to be (65 ± 10) μM.

5.4. DISCUSSION

The K_{app} value was different depending on whether the buffer was citrate or acetate. There are at least two explanations for this behavior, both of which involve a single component of the acetate buffer complexing with the enzyme. The first proposal is that acetate ion binds to the acidic form of the enzyme.

[5.6]



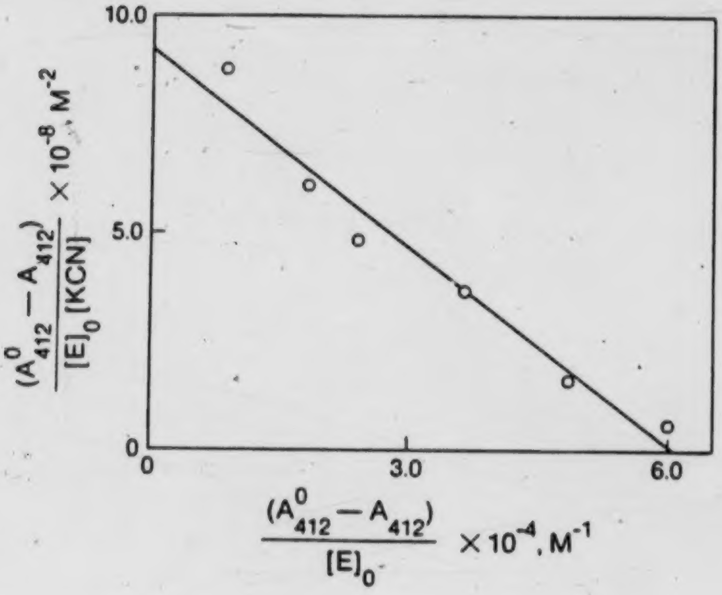


Figure 5.6. Scatchard plot of cyanide binding to PGH synthase. All reagents and conditions as in Figure 5.2. K_d = -1/slope = (65 ± 10) μM.

K_a is the actual acid dissociation constant for the heme-linked acid group and K_1 is the dissociation constant for the acetate-enzyme complex. The above scheme leads to the following expressions for k_{+app} and K_{app}:

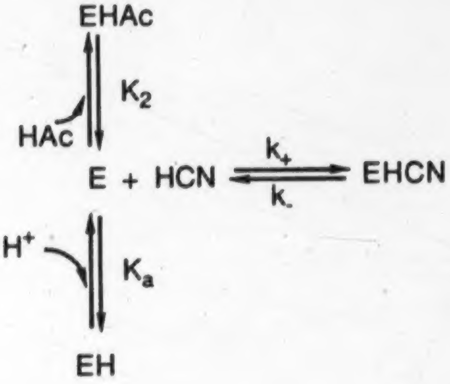
[5.4]
$$k_{+app} = \frac{k_+}{1 + \frac{[H^+]}{K_{app}}}$$

and

[5.7]
$$K_{app} = \frac{K_a}{1 + \frac{[Ac^-]}{K_1}}$$

The other possibility is that acetic acid binds to the basic form of the enzyme:

[5.8]



This gives the same expression for k_{+app} (Equation [5.4]) with K_{app} now given as:

$$K_{app} = \frac{K_a}{1 + \frac{K_a[Ac^-]}{K_{ac}K_2}} \quad [5.9]$$

Where K_{ac} is the acid dissociation of acetic acid.

Previous work on binding to chloroperoxidase (13,15) and HRP (15) with weakly acidic ligands such as acetate and azide indicate that it is the protonated form of these ligands which is the binding species. On the other hand, binding studies with ligands of high acidity such as perchlorate and nitrate to chloroperoxidase (16) and HRP (17) show that it is the anionic form which binds. With this in mind we favor the scheme shown in Equation [5.8] with the weak acid acetic acid binding to unprotonated PGH synthase, however the two proposed mechanisms are not distinguishable by kinetics.

By analogy with the results obtained with HRP and chloroperoxidase (16-18), the K_{app} determined for the citrate buffer case is assumed to be the true heme-linked acid group dissociation constant K_a for the enzyme. Previous studies with chloroperoxidase (16) and HRP (18) indicate that citrate does not bind to heme peroxidases. Knowing the K_{app} value allows one to calculate the value for K_1 and K_2 through the use of the equations derived for K_{app} (Equations [5.7] and [5.9]). If complexation of acetate ion with the acidic form of the enzyme were the mechanism, the dissociation equilibrium constant, K_1, would be 3.5×10^{-2} M. If acetic acid complexed with the basic form of the enzyme, the dissociation equilibrium constant, K_2, would be 1.3×10^{-1} M.

The pK_a of 4.15 ± 0.10 for PGH synthase in citrate buffer is the first heme-linked acid dissociation determined for the enzyme. In comparison, HRP has an important acid dissociation near the active site with a $pK_a \leq 3$ (18) which may be due to aspartate residue 43 (19). Comparisons between HRP and PGH synthase must be made with caution however especially in light of the recent sequencing studies which show no significant sequence similarity of PGH synthase with horseradish peroxidase (20,21).

The K_d of (65 ± 10) μM reported for cyanide binding at pH 8.0 in 100 μM DDC, 4°C, is significantly different than the value of 190 μM reported earlier (22). This is most likely due to the different experimental conditions which in the earlier study were pH = 7.2, 10% glycerol, 25°C (22). The spectra reported for that work were similar to that reported here for the cyanide complex in the Soret region.

The spectral shift observed at long time periods (Figure 5.2) is perhaps due to secondary binding to the enzyme resulting from the high cyanide concentrations required for observable kinetics. The effect was not seen even at very long times in the equilibrium binding experiments where low cyanide concentrations were used. In the kinetic experiments the shift was slow enough that it did not interfere with the kinetics of primary cyanide binding to the enzyme.

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CHAPTER SIX

OPTICAL SPECTRA AND KINETICS OF REACTIONS OF PROSTAGLANDIN H SYNTHASE. EFFECTS OF THE SUBSTRATES: 13-HYDROPEROXY-OCTADECA-9,11-DIENOIC ACID, ARACHIDONIC ACID, N,N,N',N'-TETRAMETHYL-*p*-PHENYLENEDIAMINE, AND PHENOL AND OF THE NONSTEROIDAL ANTI-INFLAMMATORY DRUGS: ASPIRIN, INDOMETHACIN, PHENYLBUTAZONE, AND BROMFENAC*

6.1. INTRODUCTION

The enzyme prostaglandin H synthase has been shown to be one of the key substances in the mediation of inflammation, and a key to the arachidonic acid cascade (1). Thus the mechanism of action of PGH synthase** and its control by nonsteroidal anti-inflammatory drugs, of interest in its own right, is a research topic of great practical importance (2-6).

Although both the oxygenase and peroxidase activities of PGH synthase have been recognized for some time (7-10), the optical spectral properties of compounds I and II of the enzyme have only recently been determined (11,12). In this paper, through the use of these optical spectral properties, we examine the reactivity of the lipid hydroperoxide 13-OOH 18:2 as an oxidizing substrate, and the reactivity of the following potential or established reducing substrates: phenol, phenylbutazone, TMPD, indomethacin, aspirin, Bromfenac, and arachidonic acid. In addition, we examine the effect of ethanol and the stabilizing effects on the enzyme of DDC, glycerol, and Tween 20.

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* A version of this chapter has been submitted for publication.

** Abbreviations: PGH synthase, prostaglandin H synthase (EC 1.14.99.1); 13-OOH 18:2, 13-hydroperoxyoctadeca-9,11-dienoic acid; TMPD, N,N,N',N'-tetramethyl-*p*-phenylenediamine; DDC, diethyldithiocarbamate; PGG_2, prostaglandin G_2; PPHP, 5-phenylpent-4-enyl-1-hydroperoxide; Bromfenac, 2-amino-3-(4-bromobenzoyl)benzeneacetic acid; EDTA, ethylenediaminetetraacetic acid.

6.2. MATERIALS AND METHODS

PGH synthase was purified from ram seminal vesicles using a modification of the procedure of Marnett *et al.* (13). The modification included homogenization of 300 g of ram seminal vesicles in 700 mL of 50 mM Tris buffer, pH 8.0, that contained 5 mM EDTA, 1 mM phenol and 300 μM DDC. Microsomes were isolated, solubilized with Tween 20, and applied to a 2.5 × 90 cm DE52 ion exchange column. The column was eluted with a linear gradient of 50 to 150 mM Tris buffer, pH 8.0, that contained 100 μM DDC, 0.5 mM EDTA and 0.1% Tween 20. Fractions that contained PGH synthase activity were combined and concentrated by ultrafiltration to a volume of 50 mL using an Amicon YM-30 membrane. The concentrated enzyme fractions were then subjected to gel filtration on a 5 × 90 cm Sephacryl S-300 column which had been equilibrated with 50 mM Tris buffer, pH 8.0, containing 50 μM EDTA, 0.1% Tween 20 and 100 μM DDC. Elution was performed using the same solution as was used for equilibration. Fractions that contained PGH synthase activity were again combined and concentrated by ultrafiltration, as described above, to a final volume of 25 mL. The specific activities of two hematin reconstituted PGH synthase preparations in the presence of 1 mM phenol were 46.5 and 49.0 μmol O_2 consumed/min/mg protein. The non-hematin reconstituted preparations contained 5.5 and 1.1% holoenzyme activity as estimated by cyclooxygenase activity. The enzyme was titrated with ferriprotoporphyrin IX to its endpoint as detected by cyclooxygenase activity; all kinetic and rapid-scan experiments were performed on this reconstituted enzyme.

13-OOH 18:2 was prepared with soybean lipoxygenase by the method of Graff *et al.* (14) with the following modifications. Lipoxygenase incubations were conducted in 30 mL volumes of 50 mM Tris buffer, pH 9.0, containing 450 µM ammonium octadeca-9,12-dienoate. Reactions were initiated by addition of 1.3 mL of soybean lipoxygenase (1 mg/mL) dissolved in 50 mM Tris buffer, pH 9.0. After 10 min incubation at 37°C the reaction was terminated using 7.5 mL of absolute ethanol. The solution was acidified by addition of 2 mL of 1.0 M HCl and chilled on ice. A 6 mL C_{18}-reversed phase Baker-10 column was prewashed with 25 mL absolute ethanol and 25 mL distilled water; then the sample was applied. The column was washed sequentially with 25 mL of 20% ethanol solution, 50 mL of distilled water and 10 mL of hexane. The effluents were discarded. The 13-OOH 18:2 was eluted with 10 mL of ethyl formate. Ethyl formate was removed under a stream of nitrogen and 13-OOH 18:2 was dissolved in 20 mL of methanol. The purity (≥96%) and structure of 13-OOH 18:2 was confirmed by methods previously described (14).

Arachidonic acid was obtained from Nu-Chek-Prep, phenol from Fisher or BDH Chemicals. Aspirin, indomethacin, phenylbutazone, DDC, TMPD, and soybean lipoxygenase were from Sigma. Bromfenac is a product of the A.H. Robins Co.

Stoichiometry of heme binding to PGH synthase was assayed for two different enzyme preparations by measuring the rate of oxygen consumption for each aliquot of heme added to PGH synthase in the presence of excess arachidonic acid. Total reaction volume was 3.0 mL, and it contained 50 mM Tris buffer, pH 8.0, with 0.5 mM phenol and 100 µM arachidonic acid. The hematin concentration was varied from 0 to 2 µM.

Conventional optical measurements were made using a Cary 219 spectrophotometer. Stopped-flow and rapid-scan spectra were obtained on a Photal Rapid Reaction Analyzer, Model 601. First-order rate constants were calculated by a non-linear least squares analysis carried out by computer. Oxygen consumption was measured on a Yellow Springs Instrument Oxygen Monitor, Model 53.

6.3. RESULTS

6.3.1. Hematin Titration

For preparation 1, 0.16 μM hematin reconstituted optimal oxygenase activity of 0.12 μM PGH synthase. The end point indicated [heme]/[PGH synthase dimer] = 1.43 or 0.71 [heme]/[PGH synthase monomer] (results not shown). For preparation 2, 0.57 [heme]/[PGH synthase monomer] was indicated. The average of our hematin titration results is 0.64 heme/PGH synthase monomer (9).

6.3.2. Stability of the Enzyme

Spectral analysis of the Soret region of 0.28 μM PGH synthase using the Cary spectrophotometer at 25°C indicated that inclusion of 100 μM DDC in 50 mM Tris buffer (pH 8.0) and 0.1% Tween 20 produced a stable absorption spectrum at 25°C with a molar extinction coefficient of 1.42 × 10^5 M^{-1} cm^{-1} at 410 nm. Omission of DDC from the detergent-containing buffer causes bleaching. The rate of bleaching is faster in the absence of 0.1% Tween 20 (Figure 6.1). Ethanol also acts as reducing substrate when present in sufficiently high concentration.

6.3.3. Soret Spectral Behavior During the Reaction of PGH Synthase with 13-OOH 18:2

Different behavior is observed dependent upon whether DDC or glycerol is the predominant stabilizing agent. In the presence of DDC a fast reaction occurs in which the Soret band decreases in intensity. A second phase occurs in which the absorbance remains relatively constant. Finally this is followed by a slower reaction in which the band intensity increases back to its starting point (Figure 6.2). The slower final reaction occurs with an isosbestic point at 419 nm. When the DDC is reduced to 6 µM and 26% glycerol is present, the return to native enzyme is either made slower or eliminated, depending upon the amount of peroxide present.

6.3.4. Kinetics of the Fast Phase

Stopped-flow kinetics of the fast decrease in Soret absorbance when PGH synthase is reacted with 13-OOH 18:2 were measured as a function of peroxide concentration by monitoring the absorbance change at 410 nm. Different behavior was observed depending upon whether DDC or glycerol was the principal stabilizing reagent. First the results with 100 µM DDC: exponential (pseudo-first-order) decay traces were obtained for each 13-OOH 18:2 concentration (data not shown). When the first-order rate constants were plotted *versus* 13-OOH 18:2 concentration the upper trace shown in Figure 6.3 was obtained. A peroxide-dependent region is observed at small 13-OOH 18:2 concentrations, whereas the rate constant becomes independent of peroxide concentration for large amounts of 13-OOH 18:2. In the presence of 26% glycerol and 6 µM DDC, the initial first decay occurred *via* biphasic kinetics with insufficient data to analyze the initial faster phase. Best-fit parameters obtained

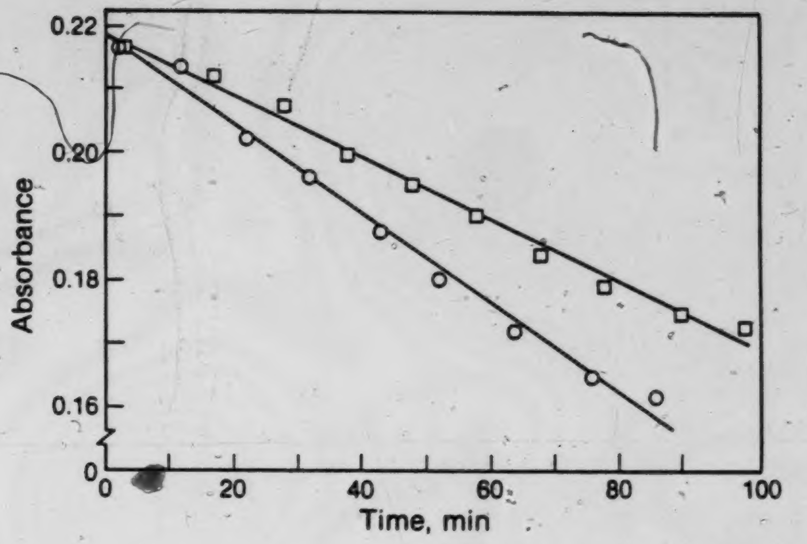

Figure 6.1. Plot of absorbance at 410 nm *versus* time when 1.5 μM PGH synthase is incubated in 50 μM Tris buffer, pH 8.0 in the presence of 26% glycerol and 6 μM DDC at 4°C with (-□-) and without (-O-) 0.1% Tween 20.

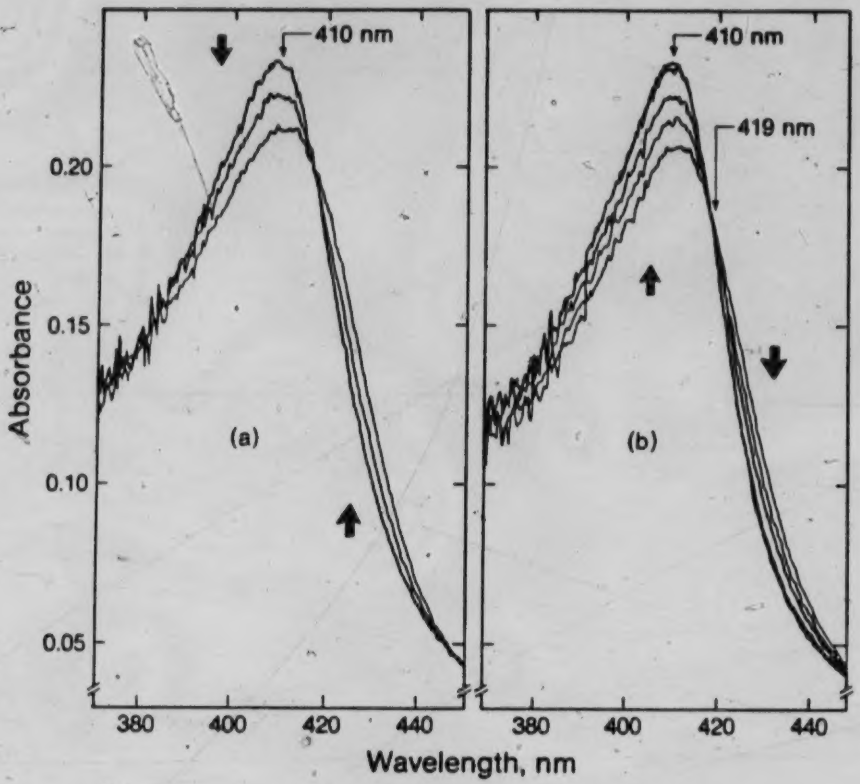

Figure 6.2. Plots of absorbance *versus* wavelength for 1.5 µM PGH synthase reacted with 30 µM 13-OOH 18:2 at 4°C in 50 mM Tris buffer, pH 8.0, 100 µM DDC, 0.1% Tween 20. (a) Rapid decrease in the Soret band 10, 20 and 40 ms after mixing. (b) Slow return of the Soret spectrum to starting conditions. From the bottom, times at which the spectra were obtained: 0.7, 1.0, 1.2, 1.7, and 2.2 s after mixing. The last spectrum was stable for several minutes.

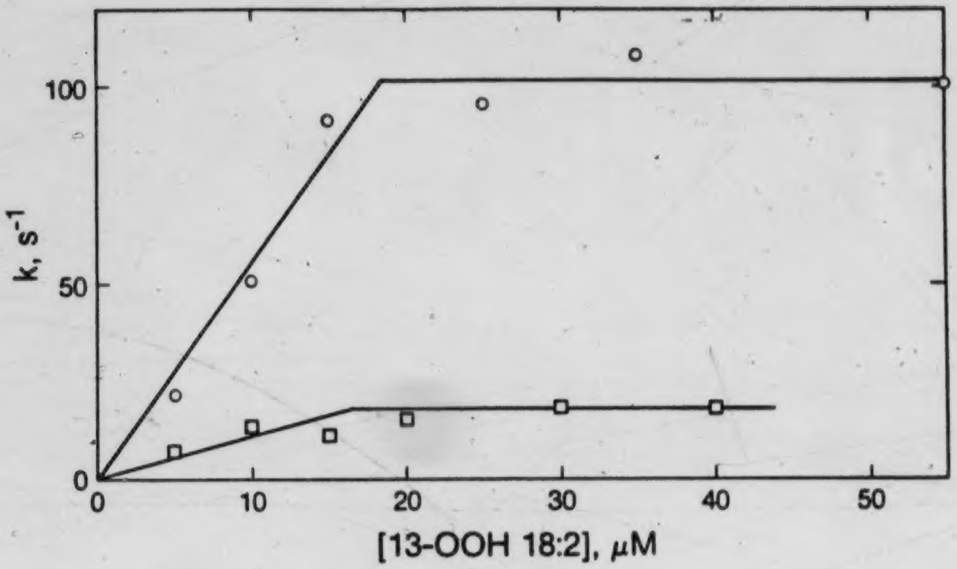

Figure 6.3. Plots of the pseudo-first-order rate constants for the fast step (absorbance decrease) when 1.5 μM PGH synthase was reacted with various [13-OOH 18:2] at 4°C in 50 mM Tris buffer, pH = 8.0 with, (-□-), 6 μM DDC, 26% glycerol and (-O-), 100 μM DDC, 0.1% Tween 20.

for the second part of the first phase led to the lower trace displayed in Figure 6.3.

6.3.5. Behavior After the Initial Decrease in Soret Absorbance

Again different behavior is observed with DDC and glycerol. In the presence of 100 µM DDC, a slow peroxide-dependent return to the spectrum of resting enzyme is observed with results shown in Figure 6.4. Starting with the minimum absorbance at 410 nm, absorbance increased sigmoidally as a function of time until the enzyme has returned to its native form. The smaller the peroxide concentration, the faster the return to the native enzyme. The results in Figure 6.4 for 60 µM and 75 µM 13-OOH 18:2 indicate that some bleaching has occurred. With 26% glycerol, 6 µM DDC, the slow return to the starting point is effectively eliminated for all but the lowest 13-OOH 18:2 concentration (Figure 6.5a).

6.3.6. Addition of Reducing Substrates

In the presence of 100 µM DDC, when both phenol and peroxide are mixed with the enzyme, conversion back to the native enzyme is accelerated. Thus phenol is competing effectively with reducing substrate already present in the solution. The dependence of the return to native enzyme upon phenol concentration is displayed in Figure 6.6.

6.3.7. The Overall Reaction of PGH Synthase with 13-OOH 18:2 and Reducing Substrate

The type of complete reaction traces obtained at 410 nm and typically observed in the present study (Figures 6.4-6.6) can be discussed in three parts.

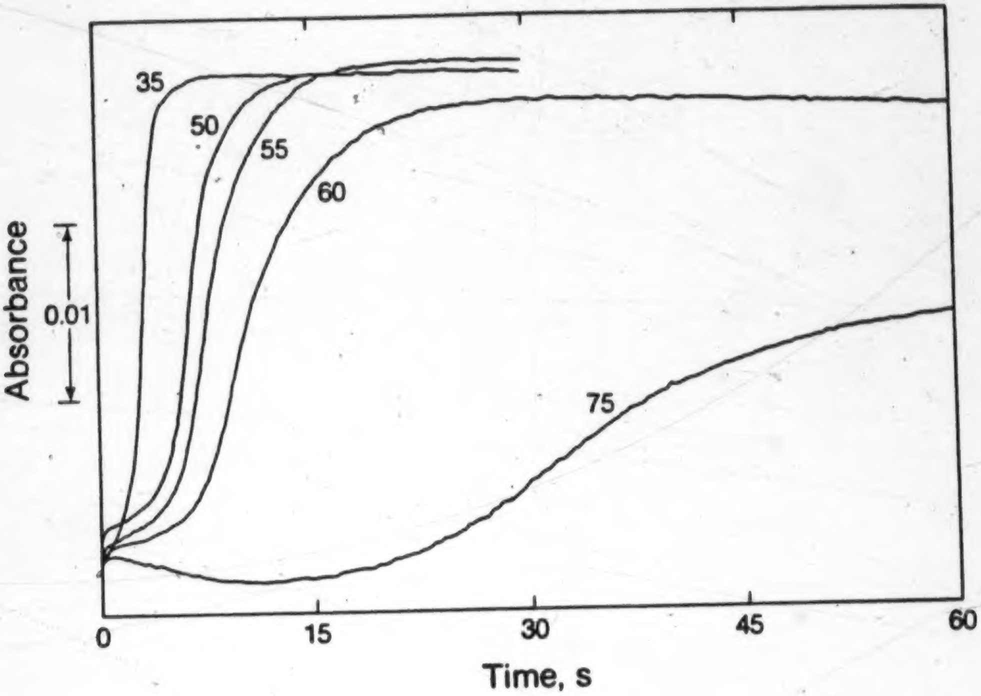

Figure 6.4. Plots of absorbance at 410 nm *versus* time for the reaction of 1.5 μM PGH synthase with varying peroxide concentrations. From the left: [13-OOH 18:2] = 35, 50, 55, 60, and 75 μM. All other reagents and conditions as in Figure 6.2.

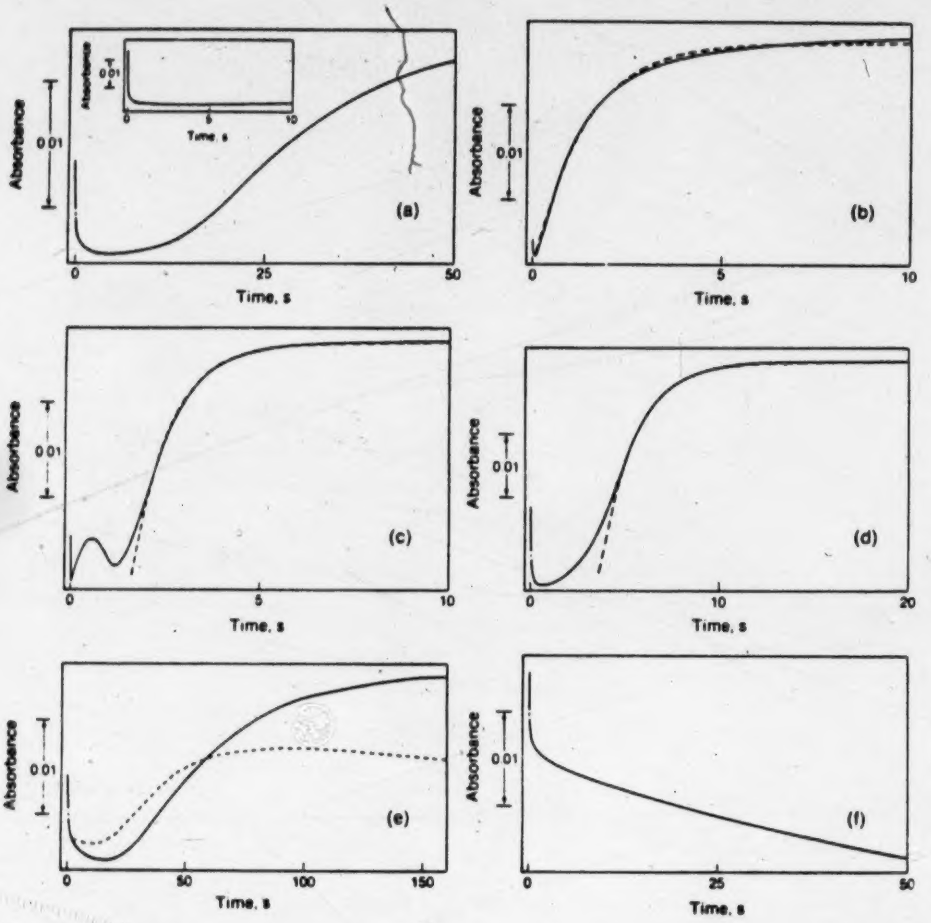

Figure 6.5. Plots of absorbance at 410 nm *versus* time for the reaction of 1.5 µM 13-OOH 18:2 in 50 mM Tris buffer, pH 8.0, 26% glycerol, 6 µM DDC at 4°C with the indicated concentration of co-substrate: (a) none, (b) 20 µM phenol, broken line is curve-fit, (c) 10 µM TMPD, broken line is curve-fit, (d) 20 µM indomethacin, broken line is a curve-fit, (e) 50 µM Bromfenac, broken line is the trace done with no co-substrate at that time scale for comparison, (f) 10 µM arachidonic acid.

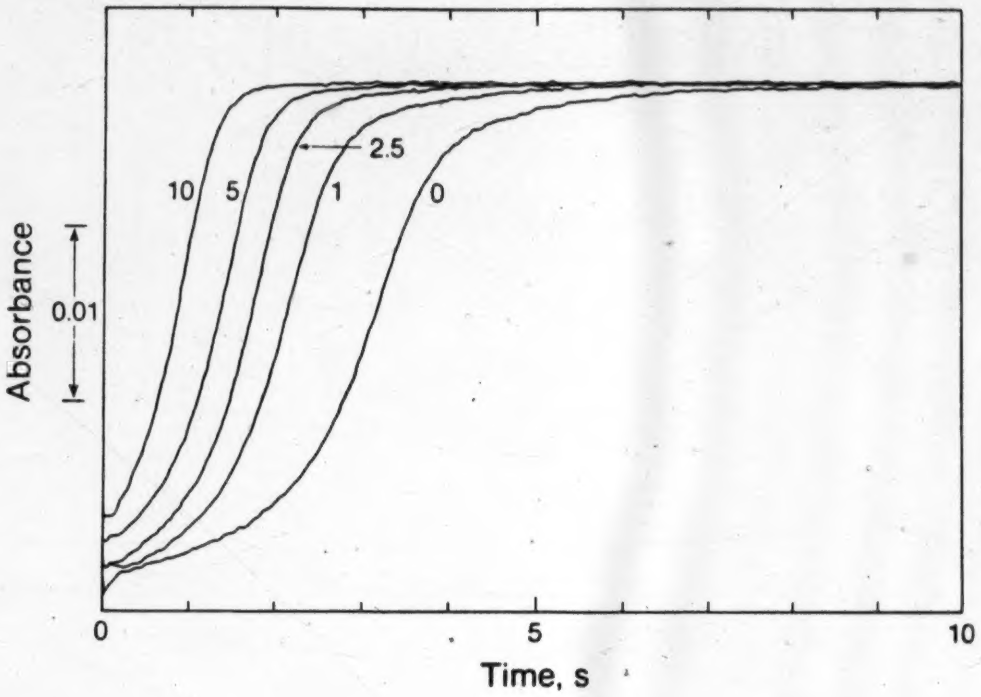

Figure 6.6. Plot of absorbance at 410 nm *versus* time for the reaction of 1.5 μM PGH synthase with 35 μM 13-OOH 18:2 and various phenol concentrations. From the right: [phenol] = 0, 1, 2.5, 5, and 10 μM. All other reagents and conditions as in Figure 6.4.

The first phase is the rapid decrease in absorbance in the presence of a fatty acid hydroperoxide such as 13-OOH 18:2 which is accelerated by the presence of reducing substrate such as phenol. This can be accounted for qualitatively by the conversion of native PGH synthase to compound I followed by a fast reduction of compound I to compound II by reducing substrate; it can be described as a pre-steady state phase.

The second phase of the reaction occurs in the region of the minima in the reaction curves. The system can be described as being a steady state. Increase in peroxide concentration increases the length of the steady state phase; increased reducing substrate concentration decreases it. Thus the enzyme is recycling with native enzyme, compound I, and compound II all in the steady state. The relative steady state concentrations are dependent upon the relative rate constants, and the dominant species in terms of concentration will be the slowest reacting component. Our results at 410 nm, combined with known spectral properties (11,12), are consistent with compound II having the highest concentration.

The third and final phase is the transient state return to native enzyme. Once the supply of hydroperoxide is exhausted then the dominant reaction is between compound II and reducing substrate. The increase in absorbance at 410 nm reflects the return of compound II to native enzyme; often a pure exponential trace is observed.

The following results all refer to solutions containing 26% glycerol and 6 µM DDC. Both reducing substrate and peroxide in one reservoir are mixed with enzyme in the other reservoir of the stopped-flow apparatus.

The presence of phenol in the reaction mixture brought about an increase in the rate of absorbance changes. First there was a decrease in absorbance which was much faster than that observed with no reducing substrate. In the absence of phenol a poor fit of an exponential decay curve indicated a rate constant of 7.4 s^{-1}. In the presence of 20 μM phenol, the exponential fit was good with a rate constant of 154 s^{-1} (Figure 6.7). These results can be explained by the following reactions; the initial reaction of PGHS + peroxide followed by the reaction of compounds I and II with the reducing agent. If the decrease in absorbance were only due to the oxidation of the enzyme by the peroxide then the rate would be the same in the presence and absence of phenol. The effect of the reducing substrate is interesting and deserves further study. The emphasis of the rest of the work described here, however, is based on the last step in the cycle, i.e., the return of the enzyme to the native state through the action of various reducing substrates.

(a) **Phenol.** The effect of phenol on the last step is dramatic (Figure 6.5b); we see a striking increase in rate.

(b) **Phenylbutazone.** The reaction of phenylbutazone was similar to that of phenol but slower (results not shown).

(c) **TMPD** shows unusual reducing ability (Figure 6.5c). The reason for the bump in the traces is not clear. It appears that in aqueous solution the TMPD becomes oxidized and the oxidized product may affect the enzyme's cycle. The color of the aqueous TMPD solution becomes darker as time goes on. The latter part of the reaction trace was analyzed by curve-fitting and the rate was faster than in the case of phenol.

(d) **Indomethacin** is a reducing agent. The exponential curve fit to the last phase of the reaction trace is very good (Figure 6.5d).

(e) **Aspirin** has absolutely no effect (results not shown).

(f) The nonsteroidal anti-inflammatory drug Bromfenac has an unusual effect. It does not act like the reducing agents. It slows steps two and three of the overall reaction (Figure 6.5e) and protects the enzyme from denaturation

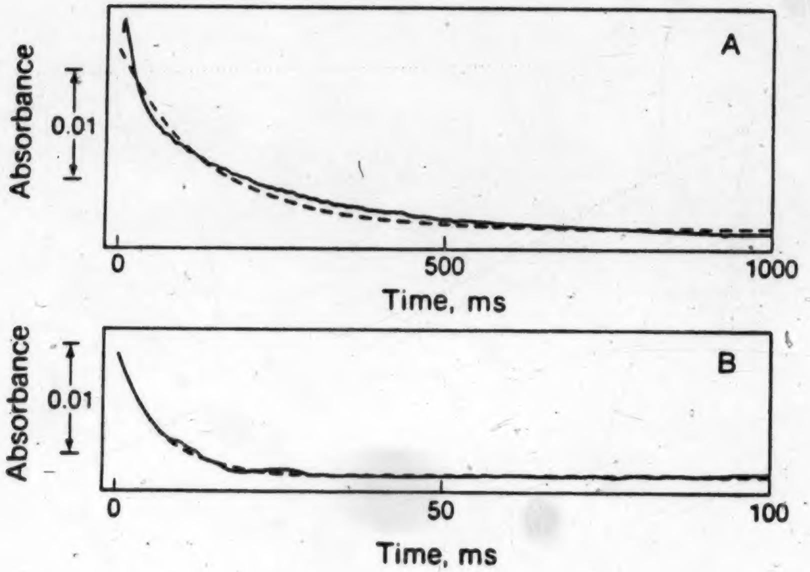

Figure 6.7. Plot of absorbance at 410 nm *versus* time with no added substrate in A and 20 µM phenol in B, all other reagents and conditions as in Figure 6.8. Broken line is an exponential curve-fit.

during the third step. On longer time scales in the absence of Bromfenac, the absorbance decreases indicating denaturation, whereas in the presence of Bromfenac the absorbance continues to increase indicating return of compound II to native enzyme without denaturation.

(g) **Arachidonic acid** causes a decrease in absorbance. It may be that the arachidonic acid reacts with compound I to form PGG_2 and that the subsequent reaction with the product PGG_2 causes bleaching of the enzyme (Figure 6.5f). Similar behavior was observed when the enzyme was reacted with excess 13-OOH 18:2.

Ratings for reducing ability (best to worst) based on observation of the transient state (last) step in the observed absorbance changes are as follows: TMPD > phenol > indomethacin > phenylbutazone >> aspirin. Where possible pseudo-first-order rate constants were calculated for the third phase of the enzyme cycle (Table 6.1). Four of these compounds were among those tested using steady state kinetics (15); our results agree with the exception of indomethacin which has been assayed to have no reducing substrate ability in the previous work (15). The nonsteroidal anti-inflammatory drug Bromfenac appears to have both an inhibitory and protective effect whereas arachidonic acid appears to cause bleaching.

6.4. DISCUSSION

The reaction products of the arachidonic acid cascade have been intensively studied for the past 30 years. For a review see reference (16). As is usually the case in complex biological systems the enzymology has lagged far behind. PGH synthase catalyzes the committed step in the biosynthesis of prostaglandins and a picture of its mechanisms of action has been emerging slowly (15,17-24). The peroxidase activity of PGH synthase is best induced by

Table 6.1. First-Order Rate Constant for Third Phase of the Enzyme Cycle with Various Reducing Substrates*

Reducing Substrate	k ± standard deviation, s^{-1}
-	0.05 ± 0.01
Phenylbutazone	0.12 ± 0.01
Indomethacin	0.17 ± 0.02
Phenol	0.50 ± 0.01
TMPD	0.9 ± 0.2

*50 µM Tris buffer, [DDC] 6 µM, 26% glycerol, pH 8.0, [PGH synthase] 1.5 µM, [13-OOH 18:2] 5 µM, [reducing substrate] 10 µM, temperature 4°C.

fatty acid hydroperoxides or PPHP (25); hydrogen peroxide tends to react more slowly with the enzyme (26).

Our transient state kinetic results indicate that the spectral properties of PGH synthase can be utilized to determine relative reducing abilities of various substrates. The rate of return of compound II of PGH synthase back to the native state is dependent upon reducing substrate and can be measured by stopped-flow kinetics. The rate constants are compared for various substrates (Table 6.1).

The three regions of kinetic traces (Figure 6.5a) can be summarized as follows. In the first phase (decrease in absorbance at 410 nm), the native enzyme is in the process of being converted to a steady state mixture of native enzyme and compounds I and II. In the second phase (fairly constant absorbance) the steady state is attained. In the third phase (increase approaching the original absorbance) the source of oxidizing substrate is used up; and the dominant reaction is between compound II and reducing substrate to reform native enzyme. Bleaching is a complication which affects mainly the latter two phases.

In the first phase of the reaction the rate of the shift to the steady state is dependent on the reducing substrate concentration as seen by the two different curves in the presence of 100 µM and 6 µM DDC (Figure 6.3). This approach to steady state is also accelerated by the oxidizing substrate in the cycle, 13-OOH 18:2. When peroxide concentration is sufficiently large, saturation is observed (Figure 6.3). In Figure 6.4 the ability of increased 13-OOH 18:2 to lengthen the steady state reaction with DDC is demonstrated. The higher the concentration of 13-OOH 18:2 the longer the steady state (second) phase. In Figure 6.6, the competitive reducing ability of phenol compared to DDC is demonstrated (second and third phases). Thus it should be possible to establish a relative scale of reactivities of reducing substrates even in the presence of DDC.

The ability of phenol to protect against peroxide-induced enzyme bleaching and loss of cyclooxygenase activity (27,28) can be explained most simply in terms of a decrease in transient concentration of compound II through an acceleration of the rate-limiting reaction step, compound II → native enzyme (15). The oxidized intermediate compound is capable of reaction with other molecules in the absence of added reducing substrate and it may itself be more susceptible to denaturation. In this regard the effect of Bromfenac (Figure 6.5e) is of interest, since this agent appears to protect the enzyme from peroxide-induced bleaching by a mechanism different from that of phenol.

Lipid peroxides are important in the regulation of prostaglandin biosynthesis. They serve as both prostaglandin H synthase substrates and inhibitors (29). In Figure 6.4 the dual nature of the peroxide 13-OOH 18:2 reaction is clearly illustrated. Increases in peroxide concentration cause an increase in the steady state phase of the enzyme cycle; however at the highest peroxide concentrations bleaching also occurs. Severe bleaching was observed when arachidonic acid was included with peroxide in the reaction with the enzyme (Figure 6.5f). This is enzyme inactivation (29,30). An overabundance of either peroxide or arachidonic acid causes enzyme bleaching which would certainly shut down prostaglandin biosynthesis *in vivo* which could be looked upon as a built-in anti-inflammatory response. It is unlikely, however, that this bleaching mechanism is effective for tissue protection *in vivo* in light of the severe toxicity reported for arachidonic acid (31) and the well established toxicity of peroxides.

Our results with aspirin confirm an earlier report that it shows no effect on peroxidase activity, despite its ability to acetylate PGH synthase and to inhibit cyclooxygenase activity (32).

Phenylbutazone is a nonsteroidal anti-inflammatory drug which has been shown to activate PGH synthase catalyzed epoxidation of the established carcinogen 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (33). This process is believed to be the activation step of this molecule's carcinogenicity. In the proposed mechanism phenylbutazone undergoes a one-electron oxidation *via* the peroxidase pathway. The resulting radical traps molecular oxygen to form a peroxyl radical which is the epoxidizing agent for 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene. Our results confirm the ability of phenylbutazone to serve as a reducing substrate for PGH synthase; however we found the rate of reaction to be slow in comparison to phenol, TMPD, and indomethacin (Table 6.1). The slow rate of reaction suggests that this mode of carcinogen activation would not be dominant in the presence of other, more readily oxidizable substrates. Because indomethacin is a moderately good reducing substrate one wonders if its radical could play some role in the reported renal and gastric toxicity of the compound (34).

Phenol is a very good reducing substrate for PGH synthase. It is the primary product of benzene metabolism and is believed to play a critical role together with the secondary metabolite hydroquinone in the mediation of benzene-induced myelotoxicity (35). The mechanism of this activation is thought to be brought about by a one-electron oxidation of phenol by myeloperoxidase. The resulting phenoxy radical can then oxidize hydroquinone to 1,4-benzoquinone which is the actual toxic species. It is clear from our results that a similar mechanism of phenol activation is possible through the peroxidase activity of PGH synthase. This may account for some features of benzene toxicity.

In summary, this paper illustrates some of the potential of transient state kinetics and rapid-scan spectrophotometry in elucidating the reaction pathways of PGH synthase.

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CHAPTER SEVEN

SIMILARITIES IN THE OPTICAL SPECTRA OF PROSTAGLANDIN H SYNTHASE DURING ITS CYCLOOXYGENASE AND PEROXIDASE REACTIONS*

7.1. INTRODUCTION

The enzyme PGH synthase** (EC 1.14.99.1) is responsible for catalysis of the initial steps of the prostaglandin/prostacyclin/thromboxane part of the arachidonic acid cascade (1-3). The enzyme converts arachidonic acid to PGG_2 in an oxygenation step and PGG_2 to PGH_2 in a peroxidase reaction (4,5). There have been recent reports on the spectral properties of the intermediate compounds of PGH synthase (6,7). The relationship between peroxidase and cyclooxygenase activity in this enzyme is of interest (8). In this paper we report the results of a study which compares the spectral properties of the enzyme during peroxidase and cyclooxygenase activities and discuss the implications of their similarity.

7.2. MATERIALS AND METHODS

All reagents used for buffers were of the highest grade available. Arachidonic acid was purchased from Nu-Chek-Prep; PPHP from Oxford Biomedical Research, Inc.; DDC from Sigma Chemical Co.; phenol from British Drug House; DE53 ion exchange chromatography gel from Whatman; Tween

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* A version of this chapter has been submitted for publication.

** Abbreviations: PGH synthase, prostaglandin H synthase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PPHP, 5-phenylpent-4-enyl-1-hydroperoxide; DDC, diethyldithiocarbamate; Tris, tris(hydroxymethyl)methylamine; λ_{max}, wavelength of maximum absorbance.

20 from J.T. Baker Chemical Company; glycerol from Fisher Scientific. Measurements of protein content were conducted by the Bio-Rad protein assay method using bovine serum albumin as a standard. Cyclooxygenase activity of the enzyme preparations was determined by measuring oxygen consumption for the reaction of arachidonic acid with PGH synthase in the presence of 1 μM hematin, 1 mM phenol, 200 μM arachidonic acid, 0.1 M phosphate buffer, pH 8.0 at 30°C. The reaction was initiated by addition of a small volume of concentrated arachidonic acid. PGH synthase concentrations were typically 0.1 μM in these assays.

PGH synthase was isolated from sheep seminal vesicles which were obtained from a local slaughterhouse. The vesicles were frozen on dry ice within 20 minutes of slaughter. They retain full enzyme activity for at least one year if kept on dry ice. The isolation procedure is a modification of that of Marnett *et al.* (9). There are three modifications. The first is the use of a batch procedure for the application of solubilized protein to the ion exchange DE53 gel. This protein-loaded gel is then transferred from a beaker to a column and eluted according to Marnett's procedure. The second modification is the use of 5 mM rather than 10 mM Tris buffer for the solubilization of the protein and equilibration of the ion exchange gel. The last change is the use of 0.1 mM instead of 0.3 mM DDC in the elution of the enzyme from the DE53 column.

The enzyme is isolated as a roughly 50/50 mixture of apo- and holoenzyme. Full activity is restored by the addition of excess hematin. In this study no hematin was added to the enzyme. The specific activity of the purified enzyme is roughly 20 units per milligram protein in the absence of exogenous hematin. The enzyme is >95% pure according to results of sodiumdodecyl sulfate polyacrylamide gel electrophoresis.

Rapid scan and stopped-flow experiments were made on a Photal Rapid Reaction Analyzer, Model 601. Pseudo-first-order rate constants were determined by a curve-fit analysis carried out by computer. Oxygen consumption was measured with a Yellow Spring Instrument Model 53 Oxygen Monitor equipped with a Clark-type polarographic electrode.

7.3. RESULTS AND DISCUSSION

7.3.1. Reaction of PGH Synthase with PPHP in the Absence and Presence of Reducing Substrate

The Soret spectrum of PGH synthase was monitored by rapid scan spectrophotometry during its reaction with PPHP in the absence of reducing substrate. Compound I formation was observed (Figure 7.1a). The λ_{max} shifted from 412 nm to 415 nm and the absorbance dropped to 80% of its original value with an isosbestic point at 426 nm. These results are in good agreement with previous work on compound I formation in the reactions of PPHP (6) and PGG_2 (7) with PGH synthase. A stopped-flow experiment was carried out, monitoring absorbance change at 412 nm for compound I formation. Pseudo-first-order kinetics were observed with a rate constant of 250 ± 20 s^{-1}. This yields a second-order rate constant of $(1.7 \pm 0.2) \times 10^7$ M^{-1} s^{-1} if one assumes the rate law:

$$\text{Rate} = k_{app}[\text{PPHP}][\text{PGH synthase}] \qquad [7.1]$$

$$\text{Rate} = k_{obs}[\text{PGH synthase}] \qquad [7.2]$$

$$k_{app} = \frac{k_{obs}}{[\text{PPHP}]} \qquad [7.3]$$

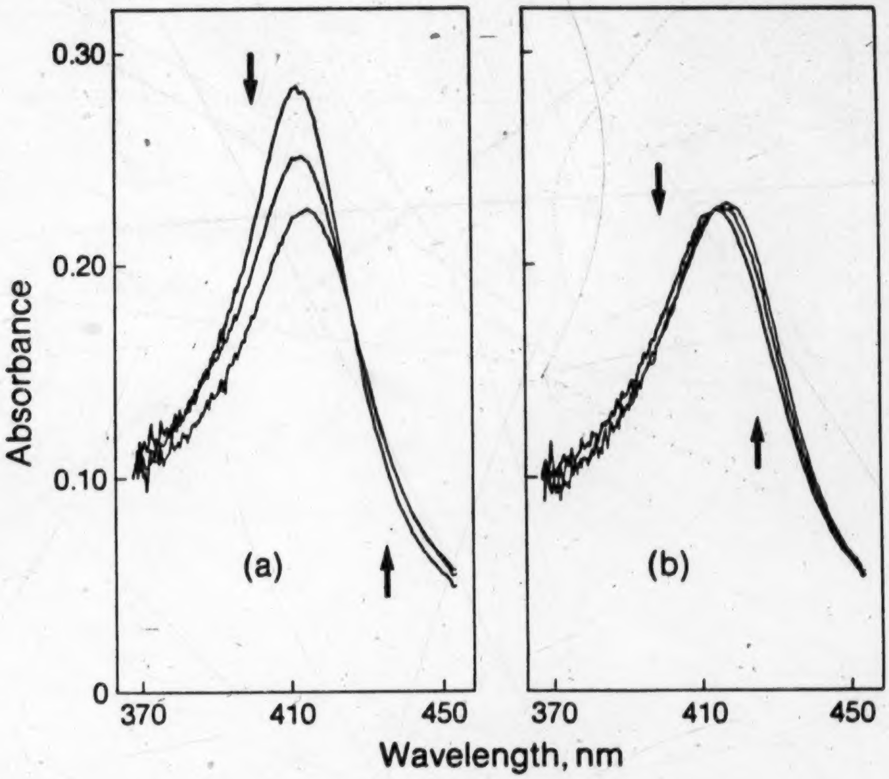

Figure 7.1. Plot of absorbance *versus* wavelength for 2.3 μM PGH synthase reacted with 15 μM PPHP at 4°C in 100 mM phosphate buffer, pH 7.7, 30% glycerol. (a) Rapid decrease in the Soret band. Highest spectrum is that of native enzyme. The next spectra were taken 1 and 16 ms after mixing. (b) Small increase in Soret band. Spectra were taken 16, 46, and 196 ms after mixing.

This value is in good agreement with the value of 1.3 × 10^7 M^{-1} s^{-1} estimated previously by rapid-scan spectrophotometry for the reaction of PPHP with the enzyme under identical experimental conditions (6). It is also close to the rate constant for the reaction of PGG_2 with PGH synthase to give compound I which was (1.4 ± 0.15) × 10^7 M^{-1} s^{-1} at 1°C in 0.1 M Tris/HCl, pH 8.1 (7).

After the formation of compound I, a second process takes place as can be seen by the rapid-scan results (Figure 7.1b). The λ_{max} shifted from 415 nm to 418 nm and the absorbance at the peak increased slightly with an isosbestic point at 414 nm. After this, bleaching takes place (results not shown).

Again the spectral changes observed in the second step are similar to results reported previously for PPHP (6) and PGG_2 (7) however the degree of increase in absorbance is much less than was previously observed for PPHP (6). The isosbestic point of 414 nm is close to the value of 412 nm that was obtained in the PGG_2 experiments (7) but not close to 400 nm which was observed in the previous PPHP work (6).

The results of these spectral investigations of enzyme intermediates in the reaction of the enzyme with PGG_2 and PPHP have been interpreted as evidence that PGH synthase behaves with a mechanism similar to that of horseradish peroxidase and other heme peroxidases (6,10,11). This mechanism involves oxidation of the native enzyme by a peroxide to the enzyme intermediate compound I which is two oxidizing equivalents above that of the native state. Compound I can then undergo a one-electron reduction upon reaction with a suitable reducing substrate to give compound II and the oxidized co-substrate. Compound II can also undergo a one-electron reduction giving another oxidized co-substrate and native enzyme.

$$\text{PGH synthase} + \text{ROOH} \rightarrow \text{PGH synthase-I} + \text{ROH} \qquad [7.4]$$

[7.5] PGH synthase-I + $AH_2 \rightarrow$ PGH synthase-II + •AH

[7.6] PGH synthase-II + $AH_2 \rightarrow$ PGH synthase + •AH

Having established the spectral properties of the enzyme in its reaction with an oxidant (which show good agreement with previous work) we then investigated the effect of reducing substrate. The rapid-scan experiments on the enzyme were repeated, this time in reaction of the enzyme with 7.5 M PPHP and 5 µM phenol.

The spectrum decreased in absorbance and then absorbance of the enzyme returned fully to its original value. No bleaching was observed; however at higher peroxide concentrations bleaching is observed (unpublished results). The spectrum first decreased in intensity with λ_{max} shifting from 412 to 415 nm and an isosbestic point at 421 nm (Figure 7.2a) followed by return of the absorbance back to that of the native state (Figure 7.2b). The fact that no compound I spectrum is observed is an indication that as soon as it is formed, it reacts very quickly with phenol to give compound II. The isosbestic point observed at 421 nm for the decrease in absorbance is an indication of only two species being present in significant amounts. Therefore this is the compound II/native enzyme isosbestic point.

7.3.2. Reaction of PGH synthase with Arachidonic Acid

The absorbance spectrum of PGH synthase was monitored by rapid-scan spectrophotometry in its reaction with arachidonic acid (Figure 7.3). The

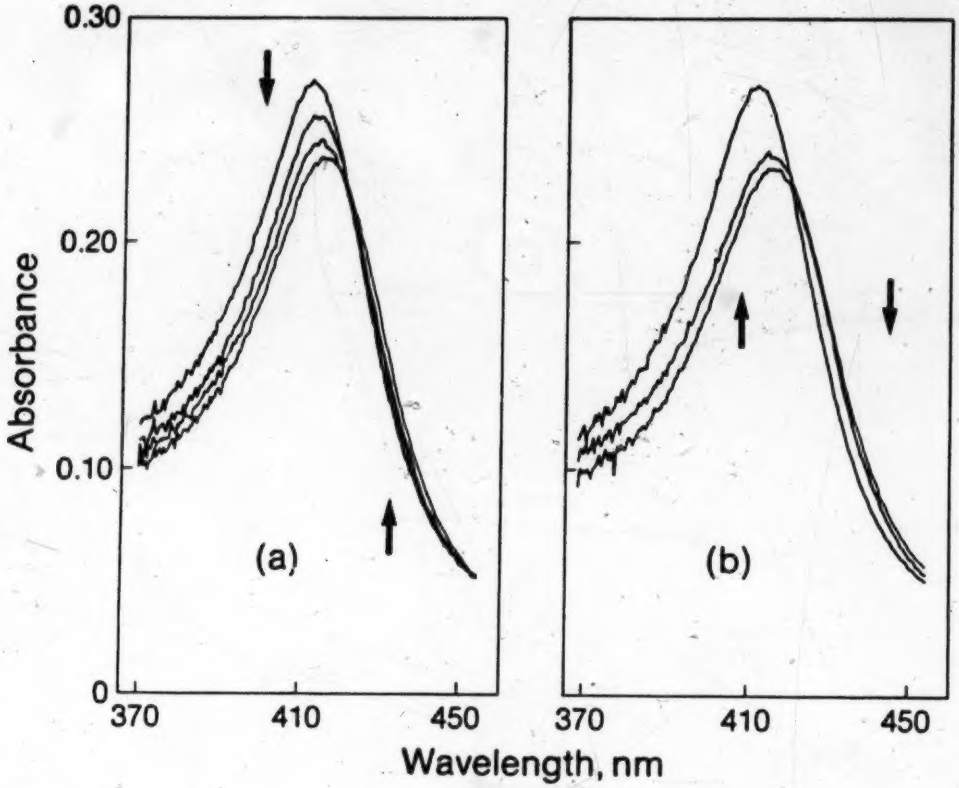

Figure 7.2. Plot of absorbance *versus* wavelength for 2.2 μM PGH synthase reacted with 7.5 μM PPHP and 5 μM phenol at 4°C in 100 mM phosphate buffer, pH 7.7, 30% glycerol. (a) Decrease in Soret band. Highest spectrum is that of native enzyme. The next spectra were taken 8, 19, and 78 ms after mixing. (b) Increase in Soret band. Starting with the lowest spectrum the timing is 42 and 286 ms after mixing. The highest spectrum is that of native enzyme which was obtained 2 minutes after mixing.

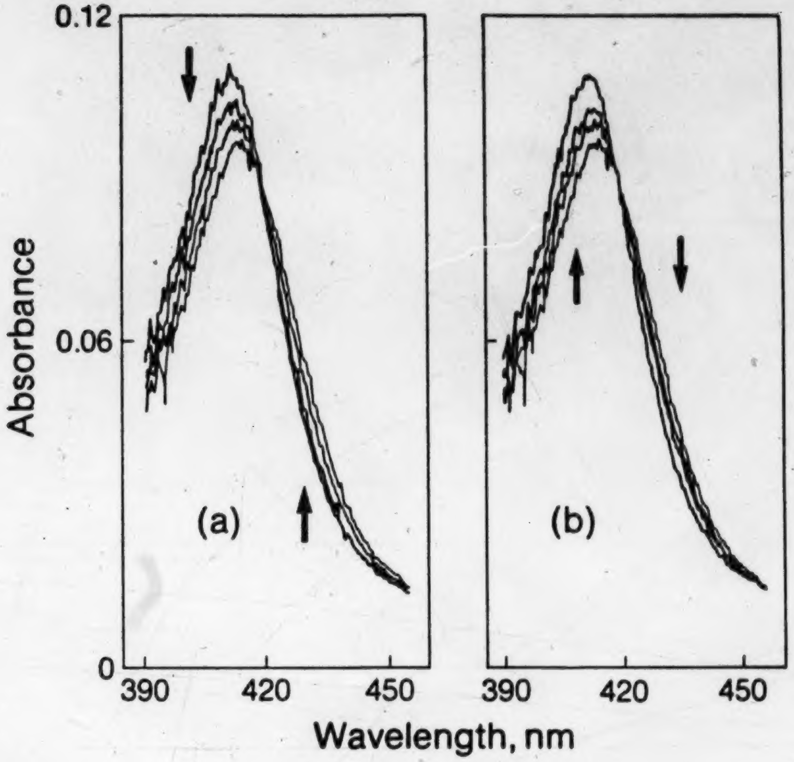

Figure 7.3. Plot of absorbance *versus* wavelength for 1.0 μM PGH synthase reacted with 12 μM arachidonic acid at 25°C in 100 mM phosphate buffer, pH 8.0, 2.0 μM DDC. (a) Decrease in the Soret band. Highest spectrum is that of native enzyme. As the peak decreases, the timing is 0.17, 0.4, and 1.6 s after mixing. (b) Increase in the Soret band. As the peak increases, the timing is 1.6, 2.2, and 3.7 s after mixing and the highest spectrum was taken 2 minutes after mixing.

reaction conditions were 1 μM PGH synthase, 100 mM phosphate buffer, pH 8.0, 20 μM DDC at 25°C. The DDC is present as a stabilizing agent. It was not possible to eliminate DDC because of extensive bleaching of the heme upon reaction of arachidonic acid with the enzyme in the absence of DDC. Spectral behavior similar to what was observed in the case of PPHP and phenol reacting with the enzyme was observed. The absorbance dropped with an isosbestic point of 420 nm (Figure 7.3a). Following that the absorbance returned to that at the native state (Figure 7.3b) again with an isosbestic point of 420 nm. A stopped-flow trace at 410 nm was taken (Figure 7.4). Results similar to these have been observed before (6,7). However in both cases only the first step was observed and the time scale for these changes was much slower. The experiments done by other workers were performed at 4°C (6) and 1°C (7) whereas in the arachidonic acid experiments of this paper the temperature was 25°C which may explain the difference.

The spectral changes observed in the reaction of peroxide and reducing substrate with the enzyme are the same as those observed in the reaction of arachidonic acid with the enzyme. Therefore the enzyme intermediates involved in the cyclooxygenation of arachidonic acid are the same as those involved in the peroxidase reaction. The oxygen consumption for this reaction was followed under identical experimental conditions as the rapid-scan (Figure 7.5) and it showed that the spectral changes observed in the enzyme are completed with the enzyme back in the native state at least 15 seconds before oxygen consumption is completely finished. This lack of a direct correlation of oxygen consumption with spectral changes on the enzyme could be an artifact due to the two different mixing techniques.

A series of experiments were carried out on the stopped-flow apparatus when 1 μM PGH synthase was reacted with different concentrations of

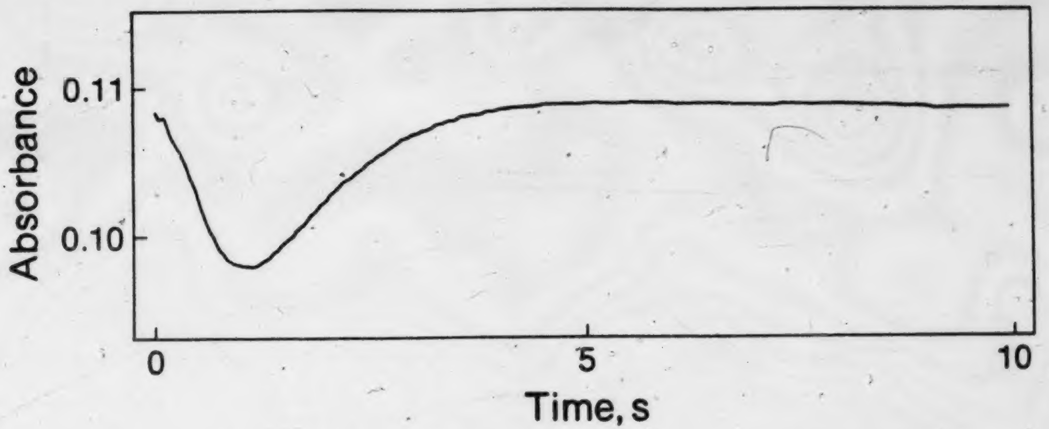

Figure 7.4. Plot of absorbance at 412 nm *versus* time for 1.0 µM PGH synthase reacted with 12 µM arachidonic acid at 25°C in 100 mM phosphate buffer, pH 8.0, 20 µM DDC.

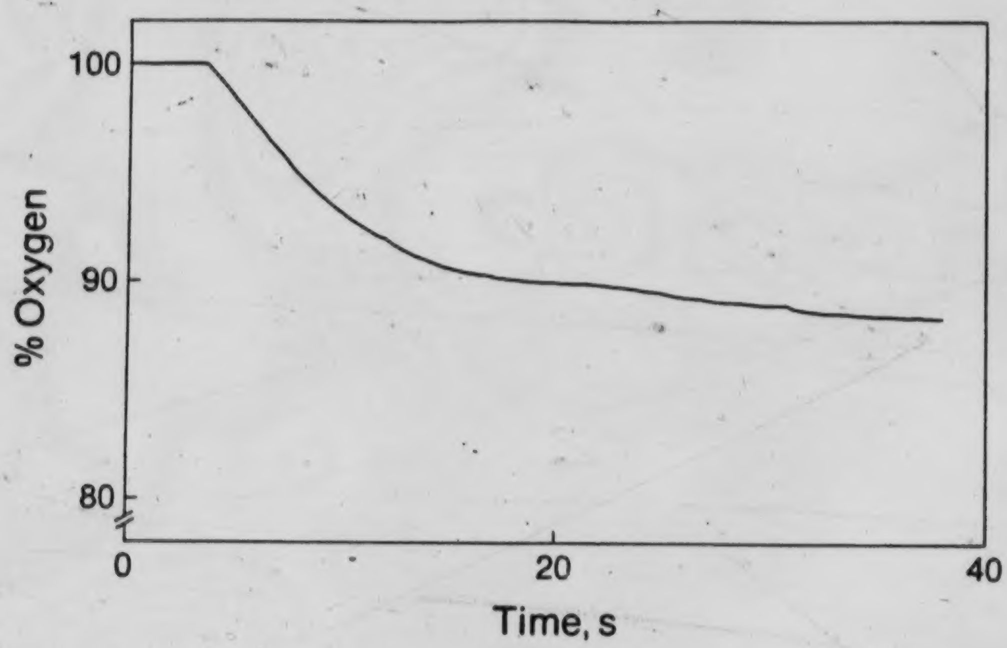

Figure 7.5. Plot of percent oxygen *versus* time for 1.0 µM PGH synthase reacted with 12 µM arachidonic acid in 100 mM phosphate buffer, pH 8.0, 20 µM DDC.

arachidonic acid (Figure 7.6). The first two traces (Figure 7.6a and Figure 7.6b) at 5 and 10 μM arachidonic acid show full recovery of the enzyme absorbance to its original value. It is also easy to see in these traces that the increase in arachidonic acid concentration has an effect on how long the enzyme takes to cycle back to the native state. This is most likely due to the increase in PGG_2 concentration that results from reaction of the arachidonic acid radical with molecular oxygen with PGG_2 during the peroxidase cycle. As arachidonic acid concentration is increased further, bleaching takes place. Because DDC is a reducing substrate for the enzyme (Chapter Six) it is not possible to attribute reduction of the enzyme intermediate in the arachidonic acid with PGH synthase solely to arachidonic acid; it is only clear that the reaction of arachidonic acid with the enzyme generates the same enzyme intermediates as that of a peroxide and reducing substrate with PGH synthase.

Another interesting link between the peroxidase and cyclooxygenase activities is that when the reaction system of 1 μM PGH synthase with 12 μM arachidonic acid is treated with 500 μM nitrosobenzene, oxygen consumption did not take place. The spin trap nitrosobenzene has been used successfully in EPR studies which confirmed that the cyclooxygenase reaction was a free radical process (12). When 20 μM PPHP was added to the nitrosobenzene treated system, oxygen consumption did occur, at a slower rate (Figure 7.7). This confirms earlier reports that peroxide is required for the cyclooxygenase activity. One possible mechanism of initiation of cyclooxygenase activity is that a hydroperoxide is generated through autoxidation of arachidonic acid. The peroxide would then start off the peroxidase cycle by reacting with PGH synthase to give compound I. The peroxide could be an impurity in the arachidonic acid; however when we pre-treated arachidonic acid with $NaBH_4$ to reduce peroxide impurities and there was no halt or delay of cyclooxygenase

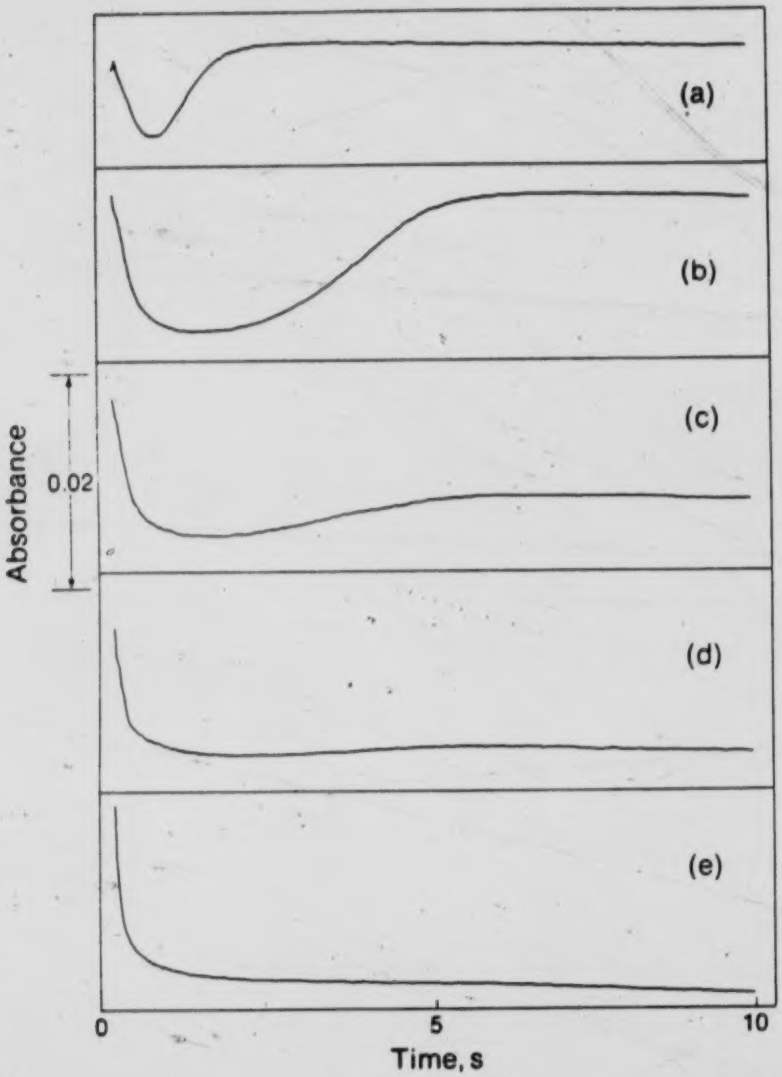

Figure 7.6. Plots of absorbance *versus* time for 1.0 µM PGH synthase reacted with arachidonic acid at 25°C in 100 mM phosphate buffer, pH 8.0, in 20 mM DDC. (a) 5 µM arachidonic acid, (b) 10 µM arachidonic acid, (c) 20 µM arachidonic acid, (d) 30 µM arachidonic acid, (e) 40 µM arachidonic acid.

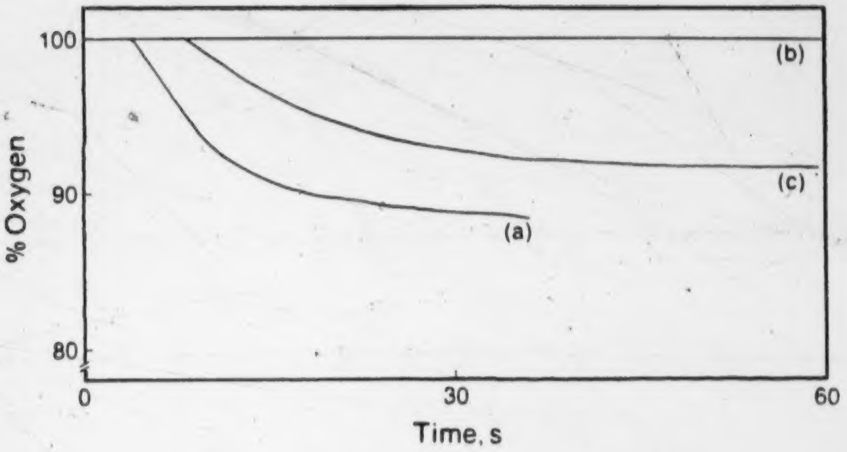

Figure 7.7. Plots of percent oxygen *versus* time for 1.0 μM PGH synthase reacted with 12 μM arachidonic acid in 100 μM phosphate buffer, pH 8.0, 20 μM DDC. (a) nothing added, (b) with 500 μM nitrosobenzene, (c) with 500 μM nitrosobenzene and 20 μM PPHP.

activity. The autoxidation may be assisted by the iron in the enzyme or heavy metal contaminants in the enzyme preparation. Work on cyclooxygenase activity of PGH synthase has shown that peroxide is required (13) and that it is a free radical process (12,14). Several authors have gone further suggesting that the cyclooxygenase activity of the enzyme is due to the type of linked oxygenase activity with compound I of the enzyme reacting with arachidonic acid to give a radical at C_{13} which then reacts with two molecules of dissolved molecular oxygen to form PGG_2 (15-18). Our results are not inconsistent with this hypothesis. We have shown that peroxidase and cyclooxygenase reactions have the same spectral intermediates and we have confirmed earlier reports that oxygen consumption is a peroxide-dependent process.

The fact that arachidonic acid reacting with PGH synthase in the absence of reducing substrate causes bleaching suggests that arachidonic acid does not react quickly with compound II of the enzyme. This may be the key role that phenol plays in the stimulation of cyclooxygenase activity of the enzyme. By reacting with compound II to give back native enzyme, phenol prevents accumulation of compound II which is unreactive to arachidonic acid and perhaps more susceptible to bleaching.

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CHAPTER EIGHT

GENERAL DISCUSSION

Work on two enzymes is presented in this thesis: the well known plant enzyme horseradish peroxidase and the recently discovered animal peroxidase, prostaglandin H synthase. The work on HRP* is on its catalysis of the oxidation of both isobutyraldehyde and malonaldehyde. The prostaglandin H synthase work began with a cyanide binding study followed by a spectral and kinetic analysis of the enzyme intermediates formed in the reaction of the enzyme with peroxide and reducing substrates. Finally the reaction of PGH synthase with arachidonic acid was examined.

The two enzymes have a number of features in common despite their different origins. Both are heme-peroxidases with remarkably similar optical-spectral properties when reacted with peroxides (1,2). They are also both peroxidases which act as oxygenases when reacted with certain substrates. In the reactions of both malonaldehyde and isobutyraldehyde with HRP, and the reaction of arachidonic acid with prostaglandin H synthase, oxygen consumption is observed. In the case of the HRP examples, it has been demonstrated that there is no direct oxygenase activity. Rather there is a pseudo-oxygenase activity in which the free radicals generated from the normal peroxidative cycle react with dissolved molecular oxygen. The reaction of arachidonic acid with PGH synthase is less clear. It has usually been termed the "cyclooxygenase activity" of the enzyme and has been considered as a separate mode of activity from the peroxidase activity. However our results and the results of some others (1-6) indicate that the cyclooxygenase mechanism may be similar to what is observed in the HRP oxygenation systems with common enzyme intermediates for both cyclooxygenase and peroxidase activities.

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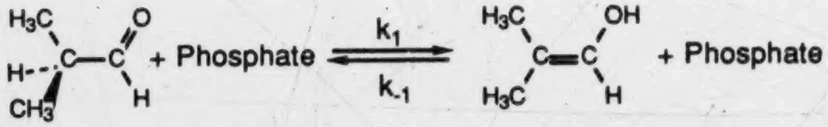
* Abbreviations: HRP, horseradish peroxidase; PGH synthase, prostaglandin H synthase; HRP-I, compound I of horseradish peroxidase; HRP-II, compound II of horseradish peroxidase; HMA, malonaldehyde.

The oxidation of isobutyraldehyde by HRP was studied. Elementary reaction rate constants were determined for the reaction of compounds I and II of HRP with the enol form of isobutyraldehyde. For the compound I reaction, the rate was $(8.0 \pm 1.0) \times 10^{6}$ M^{-1} s^{-1} and for the compound II reaction the rate was $(1.3 \pm 0.3) \times 10^{6}$ M^{-1} s^{-1}. These rates are similar to those obtained for reaction of the enzyme intermediate compounds with other good reducing substrates such as substituted phenols (7,8). This rate study allowed us to exclude the enolate anion as the reactive form of isobutyraldehyde because its rate constant was calculated to be 1.3×10^{11} M^{-1} s^{-1} which is beyond the diffusion-controlled limit (9). The fact that it is the enol and not the enolate which is reactive is consistent with what has been observed for phenols and phenolates where the unionized form is reactive (10).

We were also able to determine the equilibrium constant for keto-enol tautomerism as well as the rate constant for the forward and reverse reactions by exploiting the exclusive reactivity of compound I of HRP with the enol form of isobutyraldehyde. In the determination of the equilibrium constant, a large excess of HRP-I over the enol form of isobutyraldehyde is reacted. The rate of interconversion of keto to enol is a relatively slow process that is dependent on phosphate. The reaction involves a quick burst as compound I reacts with the equilibrium pool of enol followed by a slower reaction which is dependent on the rate of the enolization reaction. The amount of compound I reacted in the quick burst is equivalent to the amount of enol present at equilibrium. A calibration of the single beam stopped-flow apparatus allowed us to determine that concentration and thus calculate the keto-enol tautomerism equilibrium constant. The value obtained was $(1.7 \pm 0.3) \times 10^{-4}$ which is in agreement with values obtained by other experimental and theoretical methods (11-15). The value was not changed when different phosphate concentrations were employed which suggests that phosphate acts as a true catalyst.

It was possible to determine the rate of conversion of keto into enol in the tautomerism reaction by monitoring the rate of compound I disappearance. In these experiments the concentration of compound I is in excess of the equilibrium enol concentration. After the brief burst from the compound I reacting with the enol already present at equilibrium, the enolization was rate-limiting and therefore the rate constant could be determined. The measurement was made both by spectrophotometry, following the disappearance of compound I, and by monitoring oxygen consumption with a Clark-type polarographic electrode. The results were $(8.5 \pm 0.4) \times 10^{-5}$ M^{-1} s^{-1} and $(8.9 \pm 0.4) \times 10^{-5}$ M^{-1} s^{-1} which are the same within experimental error and are again in agreement with values obtained by other methods (11,15). By knowing the rate constant for the enolization, k_1, and the equilibrium constant for the tautomerism, it was possible to calculate the ketonization rate constant k_{-1} which was $(5 \pm 1) \times 10^{-1}$ M^{-1} s^{-1}.

[8.1]

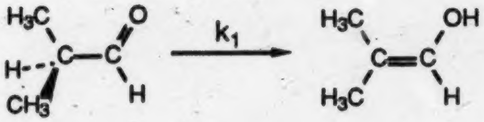


[8.2]

$$K = \frac{k_1}{k_{-1}}$$

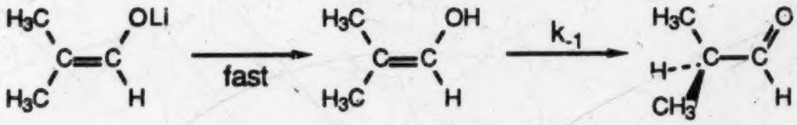
It is interesting to compare the methods of determining these rate constants. Our method involved rapid consumption of the enol. First all of the enol that was present at equilibrium was removed in a rapid burst. This was followed by a slow reaction which enol formation is the rate-limiting step.

[8.3]



In work by Kresge and his group (11,15) they prepared the lithium salt of the enol and put it in aqueous solution which immediately gives 100% enol.

[8.4]



The ketonization then proceeds with concomitant change in the UV absorbance spectrum and the kinetics of the process can be observed. The two methods approach the problem from opposite extremes in the tautomerism and thus are complementary techniques.

The concentration of enol present in these simple aldehydes is very low and therefore it is difficult to determine the equilibrium constants for the tautomerism. This method of shifting the equilibrium by consuming the small amount of enol in a specific reaction with an enzyme intermediate is thus a potentially powerful technique for studying other aspects of the keto-enol tautomerism. These include topics such as the catalytic mechanism of enolization and the determination of other keto-enol equilibrium constants.

As an extension to the project we determined the keto-enol tautomerism equilibrium constants of two other simple linear aldehydes by the same method as that for isobutyraldehyde. Propanal and butanal were studied and had tautomerism equilibrium constants of 8.0×10^{-6} and 5.5×10^{-6} respectively. These values do not take into account the hydration of the aldehydes. In the case of isobutyraldehyde, work has been done to determine the hydration equilibrium constant (16,17). However in the case of propanal and butanal hydration results in the literature are not in good agreement with each other and were obtained under different experimental conditions (18,19). Therefore we present the ratio of enol over total concentration of keto and hydrate. There are no other literature values for the keto-enol tautomerism of these aldehydes.

We also determined the rate constant for phosphate catalyzed enolization and ketonization. For the enolization the rate constant was 1.5×10^{-4} M^{-1} s^{-1} for propanal and 1.0×10^{-4} M^{-1} s^{-1} for butanal and for the ketonization the value was 19 M^{-1} s^{-1} for both. There are no literature values with which to compare these results. However because our values for the equilibrium and rate constants for isobutyraldehyde are in good agreement with literature values determined by other methods we are confident that our techniques can be extended to hitherto unstudied tautomerism reactions.

The oxidation of isobutyraldehyde by HRP is a system that has been studied extensively (20,21). It has been determined in these previous studies that the oxygen consumption is due to the normal peroxidative activity of the enzyme which generates free radicals. The oxygenation reaction is a direct result of the nature of the free radical. The isobutyraldehyde free radical scavenges dissolved molecular oxygen very quickly.

Another HRP catalyzed reaction that is oxygen consuming is the oxidation of malonaldehyde. It was reported that in the presence of acetate buffer and manganese(II), malonaldehyde is oxidized by HRP and oxygen is consumed (22,23). The main emphasis of this previous work was centered on the apparent generation of singlet oxygen in the system. The postulate of the presence of singlet oxygen was based on chemiluminescence results and singlet oxygen quencher and enhancer studies. We were curious about the enzymatic mechanism of the reaction. This was another HRP catalyzed reaction in which dissolved molecular oxygen is consumed; and it raised our curiosity about the enzymatic intermediates involved and the mechanism of oxygen consumption.

We observed that oxygen consumption occurred when manganese(II) was replaced with H_2O_2 suggesting that in the original system with no exogenous peroxide present, an endogenous peroxide was being generated. The stoichiometry of the reaction was two moles of oxygen consumed per mole of H_2O_2 reacted. If peroxide is present as a driving force for the oxygenation, this suggests a normal peroxidative mechanism for the system. The reaction of the enzyme intermediate compound I with malonaldehyde had a stoichiometry of two moles of oxygen consumed per mole of compound I reacted. Spectra of this reaction under anaerobic conditions confirmed the reaction sequence:

[8.5] $$\text{HRP-I} + \text{HMA} \rightarrow \text{HRP-II} + \bullet\text{MA}$$

[8.6] $$\text{HRP-II} + \text{HMA} \rightarrow \text{HRP} + \bullet\text{MA}$$

The role of manganese(II) in the system without exogenous peroxide is clearly an interaction of the ion with the peroxidase generated radicals to give the oxidant in the system. The oxygen consumption is always limited to the amount of oxidant present in the absence of manganese(II). One hundred percent oxygen consumption only occurs when manganese(II) is present or when the concentration of H_2O_2 is sufficiently large to consume the oxygen with a 1:2 stoichiometry. Our suggestion for the role of manganese(II) is that it reduces a peroxyl radical from the reaction of malonaldehyde radical with dissolved molecular oxygen to give the peroxide. The reduction of peroxyl radicals by manganese(II) occurs with a rate of $\approx 2 \times 10^5$ M^{-1} s^{-1} (24). The peroxide then reacts with native enzyme to give compound I and the cycle is repeated. Our hypothesis was that the apparent oxygenase reaction of HRP with malonaldehyde is a conventional peroxidase reaction leading to formation of radicals which react with dissolved molecular oxygen. The hypothesis was supported by this study.

The study of prostaglandin H synthase was undertaken as a major thesis topic. The combination of studies on both prostaglandin H synthase and HRP has yielded interesting comparisons and contrasts. At first glance the two enzymes appear to have little in common. PGH synthase is an animal enzyme as opposed to HRP from the plant kingdom. The enzymes have different substrate specificities for their oxidants and some different physical properties. However they also have several common properties. Both are heme-containing peroxidases with protoporphyrin IX as the prosthetic group. The spectral properties of the enzymes are very similar upon reaction with peroxides (1,2) so they both are peroxidase enzymes. They also both appear to act as oxygenase enzymes with particular substrates.

The first problem studied after the isolation of PGH synthase from ram seminal vesicles was the kinetics and equilibria of cyanide binding. Cyanide is often used for the investigation of hemoprotein binding sites and works particularly well on those hemoproteins which have iron in the ferric state (25). The rate of cyanide binding was studied as a function of pH and from that, a heme-linked acid dissociation was discovered with a pK_a of 4.15 ± 0.10. The identity of the species on the enzyme responsible for this ionization is not known. There have been no previous reports in the literature of any other heme-linked acid dissociations. Potential groups that may be responsible include a carboxylate group from the heme itself, one of the amino acids in the immediate vicinity of the active site which is involved with the binding process, or perhaps a distant site of ionization which has some allosteric effect on the enzyme which affects reactivity.

The dissociation constant for cyanide binding was determined to be (65 ± 10) μM. The fact that cyanide binds with this affinity is an indication that the iron of the heme in the native state is not totally shielded from interaction with the enzyme's environment and that the active site is reasonably accessible. This would preclude tight ligation at both of the axial binding sites by amino acid residues. If the sixth coordination position of the enzyme were taken up by an imidazole nitrogen from a histidine residue, for example, the cyanide complex would not be formed as readily.

Our value for the dissociation constant of 65 μM is lower than the value determined previously of 190 μM however the experiments done by the other workers were done under different experimental conditions. Our results were at pH 8.0 in 100 μM diethyldithiocarbamate, 4°C which is quite different from the previous work (26) at pH 7.2, 10% glycerol, at 25°C (26).

The active form of cyanide believed to be involved with binding is the unionized HCN. The reasons for that conclusion are, first, if it were the ionized form, the binding rate constant would be very fast (4×10^8 M^{-1} s^{-1}); approaching the diffusion controlled limit (9). Second, the active binding species for cyanide binding to other heme peroxidases such as HRP (27), myeloperoxidase (28), and chloroperoxidase (29) is the unionized form. Third, the active site for prostaglandin H synthase has a hydrophobic, non-polar environment; the affinity of the enzyme for fatty acid hydroperoxides as opposed to H_2O_2 is a testament to the fact (1). It would be hard, therefore, to imagine that the anion would be able to get into the active site and bind to the iron, particularly at a rate approaching that of the diffusion controlled limit.

Clearly a protonation step is involved in the binding process. This is known because of the heme-linked acid dissociation effect seen in the pH *versus* second-order rate constant profile. Perhaps the cyanide binding to prostaglandin H synthase is analogous to the cyanide binding to HRP where the HCN protonates an amino acid group in the active site and then an iron-CN bond is formed (30).

The next area of investigation on PGH synthase was the study of the spectral changes involved in the reaction of the fatty acid peroxide 13-hydroperoxyoctadeca-9,11-dienoic acid with the enzyme and the examination of the effect of various reducing substrates on this reaction system. The spectral changes that we saw in this reaction could be accounted for in terms of a typical peroxidase-type of mechanism with peroxide reacting with the native enzyme to give compound I. Then compound I reacts with a reducing substrate to give compound II and a free radical oxidation product. Compound II can then undergo a similar reaction to reform the native enzyme and to produce another radical generated from the reducing substrate.

It was possible to evaluate the ability of compounds to serve as reducing substrates for the enzyme by examining the rate of return of the enzyme to the native state from the peroxide induced oxidized states. This process is an indication of the rate of reactivity of these reducing substrates with compound II of the enzyme because the compound II reaction is the rate-limiting step in the steady state. We know the compound II reaction is the slowest one because it is its spectrum that is present along with native enzyme in the steady state. This indicates that in the time scale of the experiments, as soon as compound I is formed it reacts and therefore its spectrum is not observed.

Another approach to the problem of evaluating reducing substrate ability for the enzyme prostaglandin H synthase has been established by Marnett *et al.* and this method involves product analysis by high performance liquid chromatography (31). The results of our ratings on reducing substrate ability agree with this method except for the results on indomethacin. Indomethacin was rated by the Marnett group as having no reducing ability; our experiments show indomethacin to be a moderately good reducing substrate.

The oxidation of substrates by PGH synthase is being proposed as one of the mechanisms by which the body metabolizes xenobiotics and in so doing activates them to toxic, carcinogenic, and/or mutagenic species (32-37). This activation by PGH synthase is believed to be caused by the one-electron oxidation that is typical of most peroxidases. Our assay for reducing ability of substrates is one method of assessing the potential for this type of activation mechanism for a particular suspected toxic species.

The reaction of PGH synthase with 5-phenylpent-4-enyl-1-hydroperoxide was examined by rapid-scan and stopped-flow spectrophotometry. The rapid-scan spectra observed were similar to what had been seen earlier under identical experimental conditions (1). The rate constant for compound I formation was determined by stopped-flow to be $(1.7 \pm 0.4) \times 10^7$ M^{-1} s^{-1} which is in good agreement with the value estimated previously (1). When the reaction was repeated in the presence of phenol different spectra were observed. The spectral behavior was identical to what was seen previously in the assay for reducing substrate ability with just compound II and native enzyme present. The effect of phenol was to change what was being observed from a single, incomplete turnover of the enzyme to a steady state situation with the enzyme going through the three peroxidase reactions. The phenol reacts with compound I so quickly that its spectrum is not observed in the steady state.

In the reaction of the enzyme with peroxide and with no added reducing substrate, bleaching of the enzyme is observed. In the reaction of the enzyme with peroxide and phenol, bleaching is eliminated. A possible explanation is that the enzyme intermediates generated upon reaction of the enzyme with peroxide are susceptible to bleaching. By the addition of an effective reducing substrate, the concentration of these intermediates is reduced. As soon as the oxidized intermediates are formed they react with phenol and go back to the native state.

When the optical spectral behavior of the reaction of PGH synthase with arachidonic acid was studied we saw the same compound II/native enzyme mixture that was seen in the reaction of peroxide and reducing substrate with the enzyme! This is surprising because the "cyclooxygenase" and "peroxidase" activities of the enzyme are considered by many to be distinct. However based on these results, one can say that the major enzyme intermediates present during both reactions are the same.

This is evidence in support of the theory that cyclooxygenase activity is a result of removal of a hydrogen atom from C_{13} of arachidonic acid by a peroxide-induced enzyme intermediate (1-6). Thus a peroxidase-like reaction is responsible for cyclooxygenase activity. The free radical that results from hydrogen atom removal would then go on to react with two molecules of dissolved molecular oxygen to give the hydroperoxy endoperoxide prostaglandin G_2. The recent work of Ruf *et al.* on the EPR of the enzyme intermediates involved in both the cyclooxygenase and peroxidase reactions indicate the presence of an enzyme bound tyrosyl radical which may be responsible for the H-atom abstraction from C_{13} (5).

SUGGESTIONS FOR FURTHER WORK

There is great potential for further research on the enzymology of PGH synthase. The question of the effect of aspirin on the enzyme should be addressed. How does acetylation (38) shut down cyclooxygenase activity? One method of probing the aspirin's effect on PGH synthase would be a cyanide binding study of the acetylated enzyme. Apparently the enzyme's cyclooxygenase activity is shut down after treatment with 100 µM aspirin for one hour (39). It would be very informative to see what effect, if any, the treatment of the enzyme with aspirin would have on the cyanide binding properties. For example, a significant decrease in affinity for cyanide would imply that aspirin is somehow blocking access to the heme iron. A pH *versus* rate constant profile of the cyanide binding to the aspirin treated enzyme would contain information on the effect of acetylation on the heme-linked acid group described earlier.

A parallel experiment would be the investigation of the spectral properties of the acetylated enzyme upon reaction with peroxides and reducing substrates. The ability to monitor the enzymatic behavior directly through the use of stopped-flow and rapid-scan techniques is bound to shed light on the PGH synthase-aspirin interaction.

Another prospect for further work is the study of the reaction of arachidonic acid with compound I of PGH synthase. The rate constant for this reaction is an important physical constant to get because it will give an indication of the validity of the proposal that it is compound I of the enzyme which abstracts the hydrogen atom from arachidonic acid (1-6). Unfortunately the enzyme intermediate is a short-lived species so conventional stopped-flow techniques cannot be used. An alternative approach would be the use of a multi-mixing apparatus. In this instrument compound I could be reacted very quickly with arachidonic acid. This technique could be extended and rate constants for many other reactions of interest between various reducing substrates and the enzyme intermediates of PGH synthase could be determined. From these data, a detailed picture of the mechanisms of both catalysis and inhibition could emerge.

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APPENDIX A

EXPERIMENTAL DETAILS

A.1. SYNTHESIS OF MALONALDEHYDE

The synthesis of the sodium salt of malonaldehyde was accomplished by a published procedure (1) with modifications suggested by Durán (2).

Tetraethoxypropane from Sigma Chemical Co. was distilled under vacuum and 3.3 g was combined with 1.2 mL of 1 M HCl and 1.6 mL H_2O and vigorously stirred for 1.4 hours. (If the hydrolysis were allowed to go for longer times, impurities in the final preparation were observed as shown by the yellowish color of the powder.) The homogeneous yellow solution was cooled on ice and the pH adjusted to 10 with 5 M NaOH. A red solution formed. To this solution, 0.8 L of acetone was slowly added and a precipitate formed. The precipitate was filtered on Whatman #42 paper, washed with acetone, and then dried under a 1 torr vacuum at room temperature for 12 hours. The salt was dissolved in a minimum volume of hot CH_3OH and then active charcoal was added. The solution was heated just below boiling for 30 minutes and then filtered on Whatman #42 paper. One liter of ether was added to the filtrate and a white precipitate formed. It was filtered and dried under a 1 torr vacuum for 24 hours. The purity of the salt was confirmed by its uv spectrum in methanol ($\lambda_{max} = 266$, $\varepsilon = 22{,}700\ cm^{-1}\ M^{-1}$) (1).

A.2. PURIFICATION OF PROSTAGLANDIN H SYNTHASE FROM RAM SEMINAL VESICLES

The purification of prostaglandin H synthase from ram seminal vesicles was carried out following a published procedure (3) with several modifications. Ram seminal vesicles were obtained from Lambco Inc., a sheep slaughterhouse in Innisfail, Alberta, and were frozen on dry ice within 15 minutes of slaughter. The vesicles were stored on dry ice and retained full enzyme activity for at least one year. For a typical enzyme isolation, 100 g of ram seminal vesicles were removed from dry ice and allowed to stand at 4°C for 30 minutes. While still partially frozen, the glands were trimmed of excess fat and connective tissue and then chopped with scissors. The glands were rinsed two times with 1.15% KCl. Following that, 0.25 M sucrose, pH = 7.4, phosphate buffer, 0.01 M was added to the chopped, washed glands to a total volume of 300 mL. The mixture was blended in a Waring Blender for one minute and then poured into a beaker. A 50-mL volume of the 0.25 M sucrose solution was used to wash out the blender. The mixture was then homogenzied with a IKA-Werk, Ultra Turrac T25 homogenizer equipped with a UT-dispersing tool at medium speed for three minutes. The homogenate was centrifuged at 25,000 × g (11,500 RPM) in a Sorvall-type GSA rotor with a Sorvall RC2-B centrifuge at 4°C for 20 minutes. The supernatant volume was typically 245 mL. It was poured through three layers of cheesecloth two times and then put in ultracentrifuge tubes and spun at 100,000 × g (35,500 RPM) in a Beckman 45T1 rotor with a Beckman L5-75 ultracentrifuge for 90 minutes at 4°C. The supernatant was poured off and the pellet was scrapped into 180 mL of 5 mM Tris-HCl buffer, pH 8.0, 1% (v/v) Tween 20, 100 μM diethyldithiocarbamate. This solution was the solubilization buffer. The mixture was stirred for 1 hour and then spun in the ultracentrifuge for 90 minutes, 4°C, at 100,000 × g. The supernatant was concentrated to 15 mL with a 200-mL capacity Amicon ultrafiltration apparatus equipped with an XM50 Diaflo ultrafiltration membrane. This concentration step took from three to six hours at a pressure of 50 psi.

While the protein solution was being concentrated, DE53 ion exchange gel from Whatman was prepared according to the manufacturer's instructions. Eighteen grams of gel were mixed with 200 mL of 5 mM Tris-HCl buffer, pH 8.0, 0.1% (v/v) Tween 20, 100 µM diethyldithiocarbamate (buffer A); and this mixture was stirred thoroughly. The pH was adjusted to 8.0 at 4°C with 1 M HCl and the mixture was then allowed to sit. The gel settled and the fine particles which remained floating in the supernatant were removed by suction along with most of the supernatant. The remaining material was mixed into a thick slurry and was poured into a column for equilibration with buffer A. This was done by washing the column of gel with buffer A for 5 hours at a flow rate of 1.6 mL/min. After washing, the pH and ionic strength of the eluant matched that of buffer A.

The equilibrated gel was removed from the column and mixed with the 15 mL of concentrated protein and allowed to stir gently for one hour under vacuum. The protein loaded gel was then put into a 30-cm long column with a 1.4-cm diameter and washed with buffer A at a flow rate of 1.6 mL/min. Fractions of 16.5 mL were collected. After 7.5 fractions were collected, a linear ionic strength gradient elution from 5 mM to 100 mM Tris buffer concentration was started. The gradient apparatus was a home-made Parr type (4) with buffer A being the starting buffer and 100 mM Tris-HCl, pH 8.0, 0.1% (v/v) Tween 20, 100 µM diethyldithiocarbamate (buffer B) as the finishing buffer (Figure A.1). The volume of buffer used for the elution was 500 mL with 250 mL each of buffers A and B. The elution, which took almost 7 hours to complete, was done overnight with the help of a Pharmacia automatic fraction collector.

All fractions were assayed for cyclooxygenase activity. The oxygen consumption due to reaction of prostaglandin H synthase with arachidonic acid was monitored at 30°C in the presence of 1 µM hematin and 1 mM phenol at pH 8.0 in 0.1 M phosphate buffer with a Yellow Springs Instrument oxygen monitor

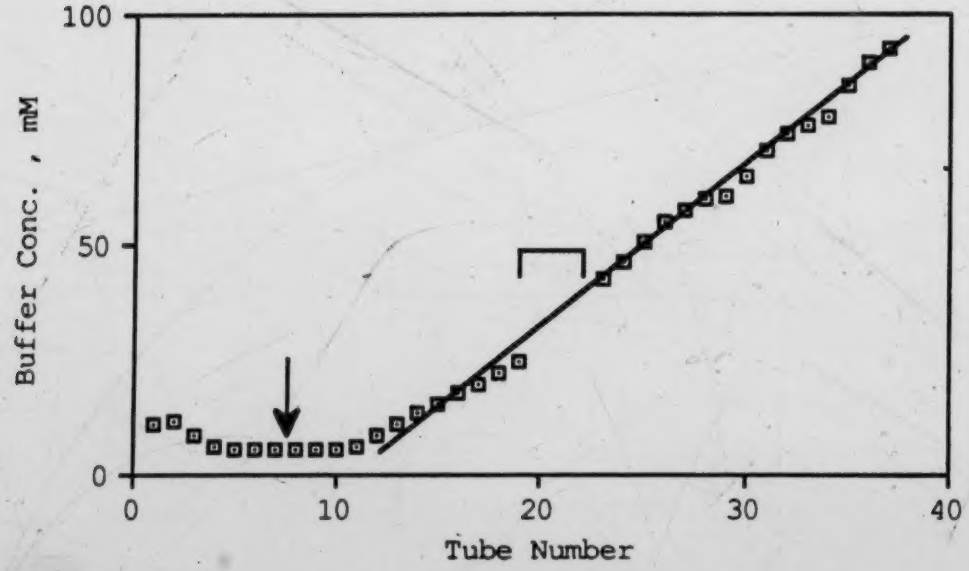

Figure A.1. Plot of Tris buffer concentration *versus* tube number for the concentration gradient employed for ion exchange chromatography elution of prostaglandin H synthase. The arrow indicates the point at which the gradient was started. The open sided rectangle indicates the tube numbers containing pure enzyme.

equipped with a Clark-type polarographic electrode. The reaction was initiated by a small volume of concentrated arachidonic acid which gave a concentration of 200 µM in the reaction mixture. The maximum rate was determined and the activity was calculated. One unit of activity is defined as 1 µmole of arachidonic acid oxidized per minute with the stoichiometry being two moles of oxygen consumed per mole of acid oxidized. Protein assays of all fractions were carried out by the spectrophotometric Bio-Rad protein assay method which uses the Coomassie Brilliant Blue G-250 protein dye. An elution profile was plotted (Figure A.2). Fractions with a specific activity of ~30 U/mg or higher were pooled and concentrated by ultrafiltration on an XM50 membrane to a volume of roughly 10 mL and then divided into 1 mL fractions and stored on dry ice. The total yield of pure enzyme was typically ~400 Units. The yield was 20% as calculated from the activity of the crude supernatant obtained after centrifugation of the gland homogenate.

A sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was carried out on the enzyme to confirm the purity (Figure A.3). The gel was run on a Mighty Small Slab Gel Electrophoresis Unit SE200 from Hoefer Scientific Instruments. The samples were prepared by heating a 50/50 mixture of sample and 0.05 M Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, 2% β-mercaptoethanol for 10 minutes at 95°C. The protein standards used were: bovine carbonic anhydrase (M.W. = 31,000 g/mol), hen egg white ovalbumin (M.W.= 42,699 g/mol), bovine serum albumin (M.W. = 66,200 g/mol), rabbit muscle phosphorylase 6 (M.W. = 97,400 g/mol), *E. coli* β-galactosidase (M.W. = 116,250 g/mol), and myosin (M.W. = 200,000 g/mol).

In addition to doing gel electrophoresis on the pure enzyme, three other protein samples from various stages in the purification were also analyzed. The crude supernatant collected after the first centrifugation, the solubilized protein

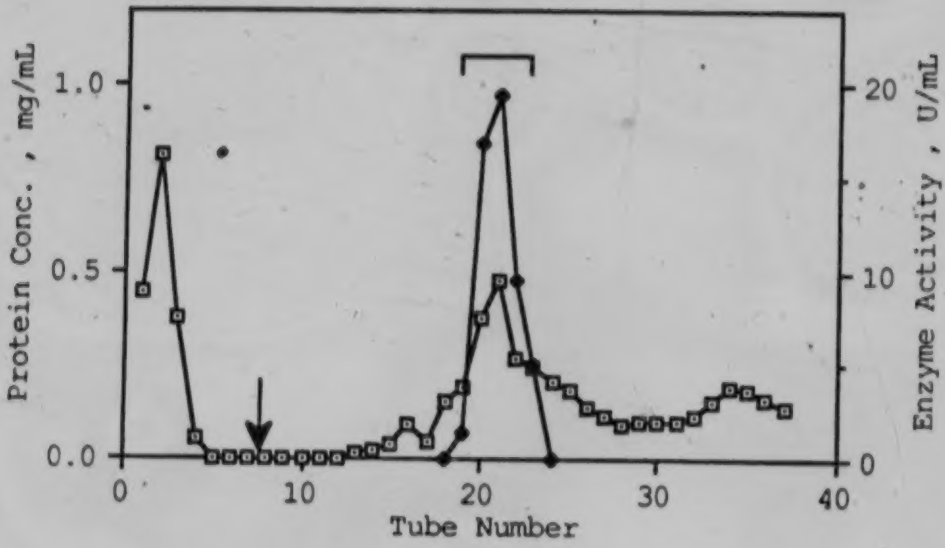

Figure A.2. Plots of both protein concentration (□) and enzyme activity (◇) *versus* tube number for the DE53 ion exchange chromatography elution of prostaglandin H synthase. The arrow indicates the point at which the ionic strength gradient was started. The open sided rectangle indicates the tube numbers containing pure enzyme.

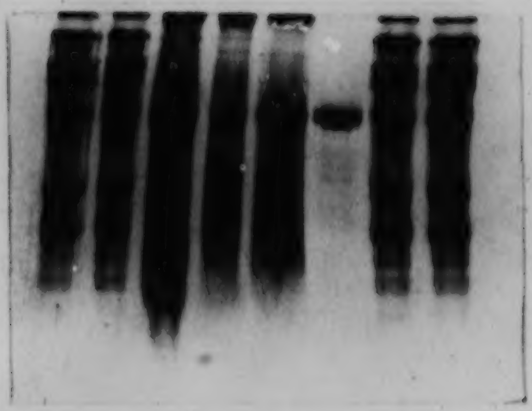

Figure A.3. SDS gel electrophoresis of protein standards (lanes 1, 2, 7, and 8), crude supernatant obtained from gland homogenate (lane 3), solubilized protein solution before the second ultrafiltration (lane 4), concentrated protein before mixing with the DE53 gel (lane 5), and pure enzyme (lane 6).

solution before the second ultrafiltration, and the concentrated protein before mixing with the DE53 gel were also subjected to gel electrophoresis. The 7.5%-20% acrylamide gradient gel was run at a constant current of 18.0 milliamps for 90 minutes. The gel was stained with a 45% methanol, 10% acetic acid, 0.35% Commassie Blue aqueous staining solution. It was destained with 30% methanol, 10% acetic acid, aqueous solution. The gel was dried according to a published procedure (5). The molecular weight of the enzyme monomer determined by SDS gel electrophoresis is 70,000 g/mol which is in agreement with previous work (Figure A.4) (6-8).

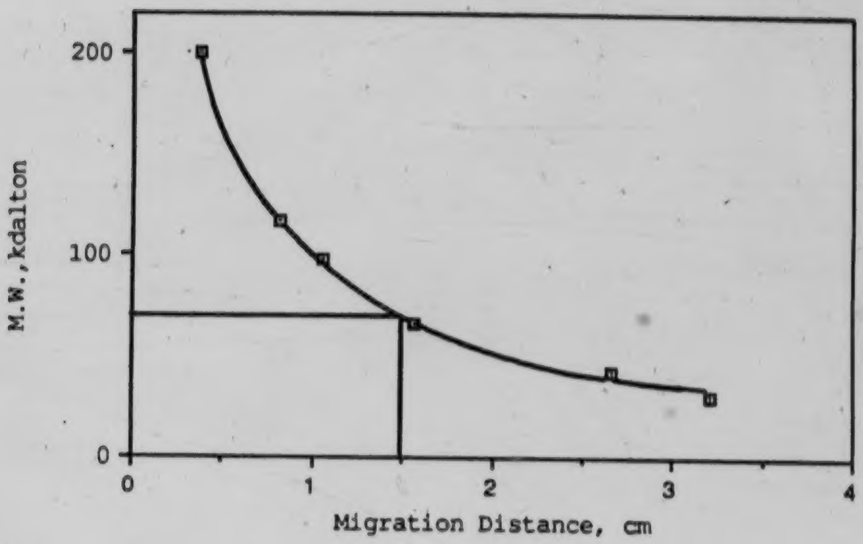

Figure A.4. Plot of molecular weight (M.W.) of the protein standards *versus* the migration distance from the SDS polyacrylamide gel electrophoresis. The lines indicate the migration distance for prostaglandin H synthase and the interpolated monomer molecular weight for the enzyme.

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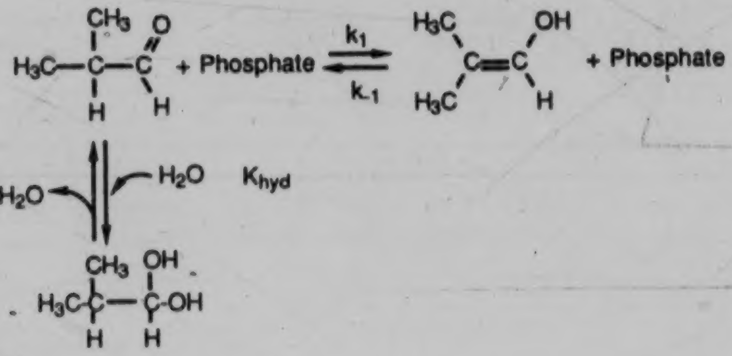
APPENDIX B

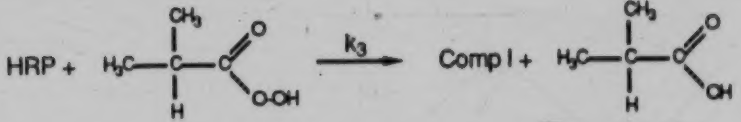
DERIVATIONS

The following two derivations are based on the mechanistic scheme:

[B.1]

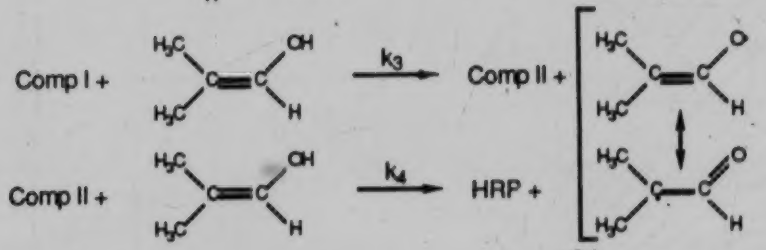

+ Phosphate
k1
k-1
+ Phosphate
H2O
H2O
Khyd

[B.2]

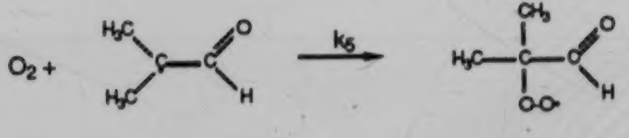

HRP +
k3
Comp I +

[B.3]

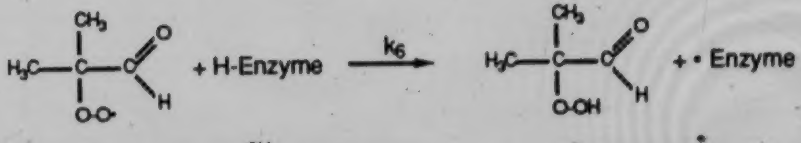
[B.4]


Comp I +
k3
Comp II +
Comp II +
k4
HRP +

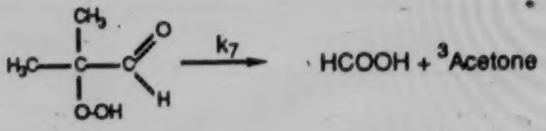
[B.5]



[B.6]


+ H-Enzyme
k6
+ • Enzyme

[B.7]


k7
HCOOH + 3Acetone

B.1. DERIVATION OF STEADY STATE RATE LAW FOR THE REACTION OF THE ENOL OF ISOBUTYRALDEHYDE AND COMPOUND I OF HORSERADISH PEROXIDASE

After the rapid reaction of the enol initially present in solution the steady state approximation can be applied to the enol concentration.

[B.8] $$\frac{d[\text{enol}]}{dt} = k_1[\text{phosphate}][\text{keto}] - k_{-1}[\text{phosphate}][\text{enol}] - k_3[\text{HRP}][\text{enol}] = 0$$

[B.9] $$[\text{enol}] = \frac{k_1[\text{phosphate}][\text{keto}]}{k_{-1}\text{p}[\text{hosphate}] + k_3[\text{HRP-I}]}$$

assuming [IBAL] ≅ [hydrate] + [keto],

[B.10] $$K_{hyd} = \frac{[\text{hydrate}]}{[\text{keto}]} ,$$

then one can write the [enol]:

[B.11] $$[\text{enol}] = \frac{k_1[\text{phosphate}][\text{IBAL}]}{(K_{hyd} + 1)(k_{-1}[\text{phosphate}] + k_3[\text{HRP-I}]}$$

The rate of reaction of HRP-I with enol is:

[B.12] $$-\frac{d[\text{HRP-I}]}{dt} = k_3[\text{HRP-I}][\text{enol}] .$$

From [B.11] and [B.12]

[B.13] $$-\frac{d[\text{HRP-I}]}{dt} = \frac{k_3 k_1[\text{phosphate}][\text{IBAL}][\text{HRP-I}]}{(K_{hyd}+1)(k_{-1}[\text{phosphate}] + k_3[\text{HRP-I}])},$$

assuming $k_{-1}[\text{phosphate}] << k_3[\text{HRP-I}]$

[B.14] $$-\frac{d[\text{HRP-I}]}{dt} = \frac{k_1[\text{IBAL}][\text{phosphate}]}{1+K_{hyd}}.$$

B.2. DERIVATION OF THE STEADY STATE RATE LAW FOR THE REACTION OF THE CARBON RADICAL OF THE ENOL OF ISOBUTYRALDEHYDE WITH DISSOLVED MOLECULAR OXYGEN

The rate of oxygen consumption is:

[B.15] $$\frac{d[O_2]}{dt} = k_5[O_2][\text{radical}]$$

By applying the steady state approximation to [radical] and [enol] an expression for [radical] can be obtained in terms of known or measurable quantities.

[B.16] $$\frac{d[\text{enol}]}{dt} = k_1[\text{phosphate}][\text{keto}] - k_{-1}[\text{phosphate}][\text{enol}] - k_3[\text{HRP-I}][\text{enol}] - k_4[\text{HRP-II}][\text{enol}] = 0$$

[B.17] $$[\text{enol}] = \frac{k_1[\text{keto}][\text{phosphate}]}{k_{-1}[\text{phosphate}] + k_3[\text{HRP-I}] + k_4[\text{HRP-II}]}$$

[B.18] $$\frac{d[\text{radical}]}{dt} = k_3[\text{HRP-I}][\text{enol}] + k_4[\text{HRP-II}][\text{enol}] - k_5[O_2][\text{radical}] = 0$$

[B.19]
$$[\text{radical}] = \frac{(k_3[\text{HRP-I}] + k_4[\text{HRP-II}])[\text{enol}]}{k_5[O_2]}$$

From [B.15], [B.17], and [B.19]

[B.20]
$$-\frac{d[O_2]}{dt} = \frac{k_1[\text{keto}][\text{phosphate}](k_3[\text{HRP-I}] + k_4[\text{HRP-II}])}{k_3[\text{HRP-I}] + k_4[\text{HRP-II}] + k_{-1}[\text{phosphate}]}$$

assuming $k_{-1}[\text{phosphate}] << k_3[\text{HRP-I}] + k_4[\text{HRP-II}]$.

[B.21]
$$-\frac{d[O_2]}{dt} = k_1[\text{keto}][\text{phosphate}]$$

from [B.10] and

[B.22]
$$k_{enol} = \frac{[\text{enol}]}{[\text{keto}]} = \frac{k_1}{k_{-1}}$$

and again assuming $[\text{IBAL}] \cong [\text{keto}] + [\text{hydrate}]$:

[B.23]
$$[\text{keto}] \cong \frac{[\text{IBAL}]}{1 + K_{hyd}}$$

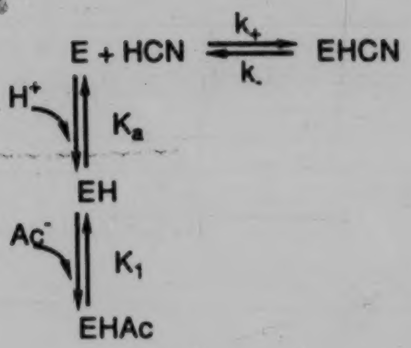
[B.24]
$$\therefore -\frac{d[O_2]}{dt} = \frac{k_1[\text{IBAL}][\text{phosphate}]}{1 + K_{hyd}}$$

B.3. DERIVATION OF EQUATIONS FOR ACETATE BINDING TO PROSTAGLANDIN H SYNTHASE

The following is a derivation of the two equations for k_{+app} which were presented in Chapter Six.

Mechanism 1: Acetate ion binds to the acidic form of prostaglandin H synthase.

[B.25]



The heme-linked acid dissociation constant, K_a, is defined as:

[B.26] $$K_a = \frac{[E][H^+]}{[EH]} .$$

The dissociation equilibrium constant for acetate ion and prostaglandin H synthase, K_1, is defined as:

[B.27] $$K_1 = \frac{[EH][Ac^-]}{[EHAc]} ,$$

and the conservation relation for enzyme concentration is:

[B.28] $$[E]_{tot} = [E] + [EH] + [EHAc] .$$

The rate of cyanide binding to prostaglandin H synthase is:

[B.29] $$\text{Rate} = k_{=app}[E]_{tot}[CN]_{tot} = k_+[E][HCN].$$

The concentration of hydrocyanic acid is effectively equivalent to the total cyanide concentration in the pH range of the study so an expression for k_{+app} can be derived in terms of k_+ by deriving an expression for [E].

First solve for [EHAc] from [B.28] and substitute into [B.27].

[B.30] $$[EHAc] = [E]_{tot} - [E] - [EH]$$

[B.31] $$K_1 = \frac{[EH][Ac^-]}{[E]_{tot} - [E] - [EH]}$$

Then solve for [EH] from [B.31] and substitute into [B.26] and solve for [E].

[B.32] $$[EH] = \frac{K_1[E]_{tot} - K_1[E]}{K_1 + [Ac^-]}$$

[B.33] $$K_a = \frac{[E][H^+](K_1 + Ac^-)}{K_1([E]_{tot} - [E]}$$

[B.34] $$[E] = \frac{[E]_{tot}}{1 + \frac{[H^+]}{K_a} + \frac{[Ac^-][H^+]}{K_aK_1}}$$

Now substitute [B.34] into [B.29] and solve for k_{+app}.

[B.35] $$\text{Rate} = k_{+app}[E]_{tot}[CN]_{tot} = \frac{k_+[E]_{tot}[CN]_{tot}}{1 + [H^+](\frac{1}{K_a} + \frac{[Ac^-]}{K_aK_1}}$$

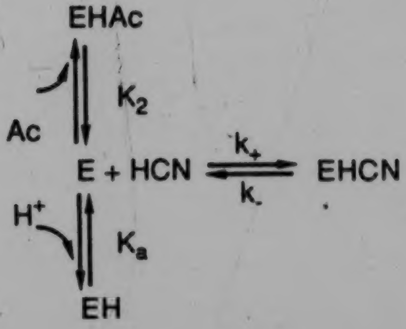
[B.36] $$k_{+app} = \frac{k_+}{1 + \frac{[H^+]}{K_{app}}}$$

with

[B.37]
$$K_{app} = \frac{K_a}{1 + \frac{[Ac^-]}{K_1}}$$

Mechanism 2: Acetic acid binds to the basic form of prostaglandin H synthase.

[B.38]



For this mechanism the dissociation equilibrium constant for acetic acid and prostaglandin H synthase, K_2, is defined as:

[B.39]
$$K_2 = \frac{[E][HAc]}{[EHAc]}$$

[B.40]
$$K_{ac} = \frac{[H^+][Ac^-]}{[HAc]}$$

Again it is necessary to solve for [E]. First solve for [HAc] from [B.40] and substitute into [B.39].

[B.41]
$$K_2 = \frac{[E][H^+][Ac^-]}{K_{ac}}$$

Then solve for [EHAc] from the conservation relation [B.20] and substitute into [B.41].

[B.42]
$$K_2 = \frac{[E][H^+][Ac^-]}{K_{ac}\Big[[E]_{tot} - [E] - [EH]\Big]}$$

Now solve for [EH] from [B.26] and substitute into [B.42] and solve for [E].

[B.43]
$$K_2 = \frac{[E][H^+][Ac^-]}{K_{ac}[E]_{tot} - \left[\,[E] - \frac{[E][H^+]}{K_c}\right]}$$

[B.44]
$$[E] = \frac{[E]_{tot}}{1 + \frac{[H^+]}{K_a} + \frac{[H^+][Ac^-]}{K_2K_{ac}}}$$

Now substituting [B.44] into [B.29] and solving for k_{+app}

[B.36]
$$k_{+app} = \frac{k_+}{1 + \frac{[H^+]}{K_{app}}}$$

with

[B.45]
$$K_{app} = \frac{K_a}{1 + \frac{K_a}{K_{ac}K_2}[Ac^-]}$$